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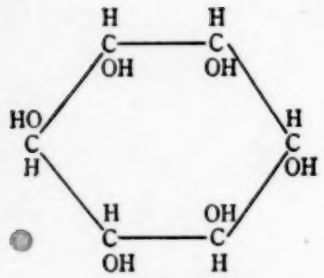

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Studies in Chemotherapy. IV. The Synthesis of Certain Unsymmetrical Disulfide Derivatives of the 4-Hydroxyphenyl Radical*,†

By SVERRIR MAGNUSSON‡, JOHN E. CHRISTIAN, and GLENN L. JENKINS

A method for the synthesis of several unsymmetrical diaryl disulfides having the 4-hydroxyphenylsulfenic radical or its acetyl or benzoyl derivatives as one of the components is described.

THE ACTIVITY of a few symmetrical disulfides against some kinds of microorganisms (1) is the basis for the synthesis of some unsymmetrical diaryl disulfides described in this work. The types of compounds herein described are limited to structures having the 4-hydroxyphenylsulfenic radical or its acetyl or benzoyl derivatives as one of the components. For the synthesis of starting materials the following methods were used.

A compound claimed to be bis(4-hydroxyphenyl) disulfide (2) was made by the reaction of sulfur with phenol under refluxing in a solution of sodium carbonate in glycerol. The method did not result in the formation of the claimed compound.

Diazotized aminophenol was reacted in the cold with potassium ethylxanthate to form the xanthate ester which upon heating and following hydrolysis with alkali yielded 4-hydroxythiophenol.

The same compound was obtained by forming the carbethoxy ester of sodium 4-hydroxyphenyl sulfonate, and following treatment of the dry sodium salt with phosphorus pentachloride, the obtained sulfonyl chloride was reduced with zinc and hydrochloric acid, and the resulting carbethoxy thiophenol was hydrolyzed with alkali to the 4-hydroxythiophenol.

By an analogous method 4-benzoxythiophenol was made. The sodium 4-hydroxyphenyl sulfonate was benzoylated; the dry sodium salt was treated with phosphorus pentachloride which, following reduction of the obtained sulfonyl chloride, yielded 4-benzoxythiophenol. This compound was

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‡ Eli Lilly and Company Research Fellow.

oxidized to bis(4-benzoxyphenyl) disulfide. The same compound was obtained by oxidation of 4-hydroxythiophenol with ferric chloride to bis(4-hydroxyphenyl) disulfide followed by esterification with benzoyl chloride. The bis(4-acetoxyphenyl) disulfide was obtained by acetylation of bis(4-hydroxyphenyl) disulfide with acetic anhydride.

The 4-acetoxy- and 4-benzoxyphenylsulfenyl chlorides, respectively, were prepared by the action of dry chlorine on the corresponding diesters of bis(4-hydroxyphenyl) disulfide dispersed in an anhydrous indifferent solvent.

The unsymmetrical disulfides were prepared as follows: By the reaction of 4-benzoxyphenylsulfenyl chloride with thiophenol and *o*-, *m*-, and *p*-thiocresols and 4-hydroxythiophenol, respectively, there were obtained 4-benzoxydiphenyl disulfide, 4-benzoxy-2′-methyldiphenyl disulfide, 4-benzoxy 3′-methyldiphenyl disulfide, 4-benzoxy-4′-methyldiphenyl disulfide, and 4-benzoxy-4′-hydroxydiphenyl disulfide. By the reaction of 2- and 4-nitrophenylsulfenyl chloride, and anthraquinone-2-sulfenyl chloride, respectively, there were obtained 2-nitro-4′-hydroxydiphenyl disulfide, 4-nitro-4′-hydroxydiphenyl disulfide, and 4-hydroxyphenyl-2-anthraquinone disulfide. The reactions of phenylsulfenyl chloride, 2-chlorobenzylsulfenyl chloride, 2,4-dichlorobenzylsulfenyl chloride, and 4-benzamido-3-chlorophenylsulfenyl chloride, respectively, with 4-hydroxythiophenol failed to yield the expected compounds.

The reaction of 2-chlorobenzylsulfenyl chloride and 4-benzamido-3-chlorophenylsulfenyl chloride, respectively, with 4-benzoxythiophenol yielded compounds which have not as yet been identified. The reaction between the 4-benzoxythiophenol and 2,4-dichlorobenzylsulfenyl chloride, and 2- and 4-nitrophenylsulfenyl chlorides, respectively, failed to yield the expected compounds. By the reaction of 4-acetoxyphenylsulfenyl chloride with 4-benzoxythiophenol the 4-acetoxy-4′-benzoxydiphenyl disulfide was obtained. The reaction of the same sulfenyl chloride with *p*-thiocresol did not result in the desired compound.

By benzoylation with benzoic anhydride in an anhydrous pyridine medium there were obtained 2-nitro-4′-benzoxydiphenyl disulfide and 4-nitro-4′-benzoxydiphenyl disulfide from 2-nitro- and 4-nitro-4′-hydroxydiphenyl disulfides, respectively, and 4-benzoxyphenyl-2-anthraquinone disulfide from 4-hydroxyphenyl-2-anthraquinone disulfide.

By acetylation with acetic anhydride the 2-nitro-4′-acetoxydiphenyl disulfide was obtained from 2-nitro-4′-hydroxydiphenyl disulfide.

EXPERIMENTAL

1. A compound claimed to be bis(4-hydroxyphenyl) disulfide was made according to the procedure of Lefevre and Desgrez (2). No analysis is reported by the authors but a melting point of 116° is listed. Recrystallized from alcohol the obtained compound melted at 119–120°.

Anal.—Calcd. for $C_{12}H_{10}O_2S_2$: S, 25.61%. Found: S, 29.69%.

2. 4-Hydroxythiophenol.—This compound was made by the Leuckart reaction (3) modified by Miller and Read (4). Yield 46 Gm. (36.5%, starting with 1 mole of 4-aminophenol and 1.4 mole of potassium ethyl xanthate), collected over a distilling range of 105–110° at 1-mm. pressure.

The same compound was more conveniently obtained according to a method described by Zincke and Ebel (5) by the following steps.

3. Sodium 4-hydroxyphenyl Sulfonate.—Ten moles of crystalline phenol were sulfonated and converted to the sodium salt according to a procedure of Paul (6). Yield 1580 Gm. (80%).

4. Carbethoxy Ester of Sodium 4-Phenol Sulfonate.—Two moles of sodium 4-hydroxyphenyl sulfonate were esterified with ethylchlorocarbonate (5). Yield 208 Gm. (73%).

5. 4-Carbethoxysulfonyl Chloride.—One mole of the carbethoxy ester of sodium 4-phenol sulfonate was converted to the sulfonyl chloride through treatment with phosphorus pentachloride (5). Yield 224 Gm. (85%); m. p. 75–76° (from glacial acetic acid).

6. 4-Carbethoxythiophenol.—Two-tenths mole of the sulfonyl chloride was reduced with zinc and hydrochloric acid (5). Yield 22.5 Gm. (56.6%). The authors report up to 70% yield from this step.

7. 4-Hydroxythiophenol.—Two-tenths mole of the 4-carbethoxythiophenol was hydrolyzed with 2 *N* sodium hydroxide (5). Yield 17.5 Gm. (69.4%) collected over a distilling range of 112–117° at 2-mm. pressure. The authors do not report the yield from this step.

The 4-benzoxythiophenol was made by the following reactions:

8. Benzoyl Ester of Sodium 4-Hydroxyphenyl Sulfonate.—One mole of sodium 4-hydroxyphenyl

sulfonate was benzoylated with benzoyl chloride (7). Yield 233 Gm. (70%).

9. 4-Benzoxyphenylsulfonyl Chloride.—One mole of the benzoyl ester of sodium 4-hydroxyphenyl sulfonate was converted to the sulfonyl chloride through treatment with phosphorus pentachloride (8). Yield 237 Gm. (80%); m. p. 114–115° (from glacial acetic acid). Schreinemakers (8) reports a m. p. of 115–116°.

10. 4-Benzoxythiophenol.—Two-tenths mole of the sulfonyl chloride was added in 5-Gm. portions to a mixture of 30 Gm. of zinc dust, 150 cc. of glacial acetic acid, and 10 cc. of concentrated hydrochloric acid, the mixture being kept below 60° during the addition. An additional 90 cc. of concentrated hydrochloric acid was added and the mixture was refluxed until a sample precipitated with water formed a clear solution with alkali. After filtering the solution was diluted with water and a white product was collected and dried. Yield 17.5 Gm. (74%); m. p. 74–75° (from ligroin).

Anal.—Calcd. for $C_{19}H_{10}O_2S$: S, 13.92%. Found: S, 13.65%.

11. 4-Benzoxyphenyl Thiobenzoate.—Made by benzoylation of 4-benzoxythiophenol with benzoyl chloride. M. p. 160–161° (from benzene).

Anal.—Calcd. for $C_{20}H_{14}O_3S$: S, 9.55%. Found: S, 9.50%.

12. Bis(4-hydroxyphenyl) Disulfide.—Two-tenths mole of 4-hydroxythiophenol was oxidized with a dilute solution of ferric chloride (3). Yield 20 Gm. (80%). Colorless needles, melting at 151–152° (from water).

13. Bis(4-benzoxyphenyl) Disulfide.—Two-tenths mole of bis(4-hydroxyphenyl) disulfide was benzoylated with benzoyl chloride. Yield 3.2 Gm. (70%).

The same compound was prepared by the oxidation of 0.1 mole of 4-benzoxythiophenol at room temperature in 50% acetic acid with 30% hydrogen peroxide. Yield 19.5 Gm. (89%). White needles from benzene; m. p. 165–166°.

Anal.—Calcd. for $C_{26}H_{18}O_4S_2$: S, 13.98%. Found: S, 14.10%.

14. Bis(4-acetoxyphenyl) Disulfide.—Two-tenths mole of bis(4-hydroxyphenyl) disulfide was esterified with acetic anhydride (9). M. p. 90–91° (from alcohol). Leuckart (3) reports m. p. 88–89°.

15. 4-Benzoxyphenylsulfenyl Chloride.—The procedure used was that described by Hubacher (9). On completion of the chlorination reaction excess chlorine was expelled from the sulfenyl chloride solution by bubbling dry nitrogen through the system for one-half hour. The sulfenyl chloride was not isolated due to rapid decomposition of the crystalline compound, but was used directly in solution for the reaction with different thiols.

All the other sulfenyl chlorides used in the following reactions were prepared in the same manner, starting with the corresponding symmetrical disulfides.

16. 4-Benzoxydiphenyl Disulfide.—One-hundreth mole of 4-benzoxyphenylsulfenyl chloride dissolved in 25 cc. of dry carbon tetrachloride was added slowly at room temperature to a solution of 0.01 mole of thiophenol in 25 cc. of dry carbon tetrachloride, followed by refluxing the mixture for one-half hour. A trace of copper bronze was used as a catalyst. Spontaneous evaporation of the solvent left a compound which, recrystallized from ligroin, melted at 103.5–104.5°. Yield 2.8 Gm. (82.8%).

The same procedure was used for the other coupling reactions.

The 4-benzoxydiphenyl disulfide was also prepared by the reaction of the sulfenyl chloride (0.01 mole) in dry benzene solution with the sodium salt of thiophenol, obtained by the reaction of metallic sodium with thiophenol in dry benzene and nitrogen atmosphere. Yield of 4-benzoxydiphenyl disulfide was 2.57 Gm. (76%).

Anal.—Calcd. for $C_{19}H_{14}O_2S_2$: C, 67.71%; H, 4.14%; S, 18.93%; Found: C, 67.52%; H, 4.17%; S, 18.94%.

17. 4-Benzoxy-2′-methyldiphenyl Disulfide.—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with 0.01 mole of *o*-thiocresol in dry chloroform. Tiny snow-white needles, m. p. 68.5–69.5° (from methanol). Yield 2.53 Gm. (70.9%).

Anal.—Calcd. for $C_{20}H_{16}O_2S_2$: C, 68.15%; H, 4.57%; S, 18.19%. Found: C, 68.10%; H, 4.85%; S, 17.97%.

18. 4-Benzoxy-3′-methyldiphenyl Disulfide.—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with an equimolar amount of *m*-thiocresol in dry chloroform. Fine snow-white needles, m. p. 75–76° (from methanol). Yield 2.35 Gm. (66.7%).

Anal.—Calcd. for $C_{20}H_{16}O_2S_2$: S, 18.19%. Found: S, 17.97%.

19. 4-Benzoxy-4′-methyldiphenyl Disulfide.—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with 0.01 mole of *p*-thiocresol in dry carbon tetrachloride. Fine snow-white needles, m. p. 115.5–116.5° (from alcohol or ligroin). Yield 2.73 Gm. (80%).

Anal.—Calcd. for $C_{20}H_{16}O_2S_2$: S, 18.19%. Found: S, 17.97%.

20. 4-Benzoxy-4′-hydroxydiphenyl Disulfide.—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with 0.01 mole of 4-hydroxythiophenol dissolved in 50 cc. of anhydrous ether. A white microcrystalline compound, m. p. 129 5–130° (from methanol). Yield 2.52 Gm. (71.2%).

Anal.—Calcd. for $C_{19}H_{14}O_2S_2$: S, 18.09%. Found: S, 17.42%.

21. 4-Hydroxyphenyl-2-anthraquinone Disulfide.—One-hundreth mole of anthraquinone-2-sulfenyl chloride was reacted with 0.01 mole of 4-hydroxythiophenol dissolved in 50 cc. of anhydrous ether. Small golden-yellow crystals, m. p. 192–193° (from glacial acetic acid). Yield 2.9 Gm. (79.6%).

Anal.—Calcd. for $C_{20}H_{12}O_3S_2$: S, 17.05%. Found: S, 17.07%.

22. 2-Nitro-4′-hydroxydiphenyl Disulfide.—One-hundredth mole of 2-nitrophenylsulfenyl chloride was reacted with 0.01 mole of 4-hydroxythiophenol dissolved in 50 cc. of anhydrous ether. Yellow needles, m. p. 137–138° (from 80% methanol). Yield 1.98 Gm. (71%).

Anal.—Calcd. for $C_{12}H_9O_3NS_2$: S, 22.95%. Found: S, 23.01%.

23. 4-Nitro-4′-hydroxydiphenyl Disulfide.—One-hundredth mole of 4-nitrophenylsulfenyl chloride was reacted with 0.01 mole of 4-hydroxythiophenol dissolved in 50 cc. of anhydrous ether. Yellow pearly platelets, m. p. 118–119° (from 80% methanol). Yield 2.15 Gm. (76.9%).

Anal.—Calcd. for $C_{12}H_9O_3NS_2$: S, 22.95%. Found: S, 22.74%.

24. 4-Acetoxy-4′-benzoxydiphenyl Disulfide.—One-hundredth mole of 4-acetoxyphenylsulfenyl chloride was reacted with 0.01 mole of 4-benzoxythiophenol in dry benzene. White microcrystalline compound, m. p. 99–100° (from ligroin). Yield 3.10 Gm. (78.2%). The same compound was prepared by the acetylation with acetic anhydride of 4-benzoxy-4′-hydroxydiphenyl disulfide.

Anal.—Calcd. for $C_{21}H_{16}O_5S_2$: S, 16.17%. Found: S, 15.68%.

25. 2-Nitro-4′-benzoxydiphenyl Disulfide.—This compound was prepared by refluxing equimolar amounts of 2-nitro-4′-hydroxydiphenyl disulfide and benzoic anhydride in anhydrous pyridine for five minutes. The reaction mixture was poured into ice water, and the benzoic acid was extracted from the precipitate with warm alcohol. Bright yellow platelets, m. p. 113–114° (from a mixture of equal parts of benzene and ligroin).

Anal.—Calcd. for $C_{19}H_{13}O_4NS_2$: S, 16.72%. Found: 17.06%.

26. 4-Nitro-4′-benzoxydiphenyl Disulfide.—From 4-nitro-4′-hydroxydiphenyl disulfide by the procedure described in Experiment 25. Faintly yellow large crystals, m. p. 124.5–125.5° (from benzene-ligroin).

Anal.—Calcd. for $C_{19}H_{13}O_4NS_2$: S, 16.72%. Found: S, 16.67%.

27. 4-Benzoxyphenyl-2′-anthraquinone Disulfide.—From 4-hydroxyphenyl-2-anthraquinone disulfide by the procedure described in Experiment 25. Yellow microcrystalline compound, m. p. 155.5–156.5° (from benzene-ligroin).

Anal.—Calcd. for $C_{27}H_{16}O_4S_2$: S, 13.68%. Found: S, 13.68%.

28. 2-Nitro-4′-acetoxydiphenyl Disulfide.—From 2-nitro-4′-hydroxydiphenyl disulfide by acetylation with acetic anhydride in the presence of sodium acetate. Faintly yellowish green needles, m. p. 66–67° (from benzene-ligroin).

Anal.—Calcd. for $C_{14}H_{11}O_4NS_2$: S, 19.95%. Found: S, 19.82%.

SUMMARY

1. A method for the synthesis of unsymmetrical diaryl disulfides has been described.

2. The following new unsymmetrical diaryl disulfides have been synthesized: 4-benzoxydiphenyl disulfide; 4-benzoxy-2′-methyldiphenyl disulfide; 4-benzoxy-3′-methyldiphenyl disulfide; 4-benzoxy-4′-methyldiphenyl disulfide; 4-benzoxy-4′-hydroxydiphenyl disulfide; 4-hydroxyphenyl-2-anthraquinone disulfide; 2-nitro-4′-hydroxydiphenyl disulfide; 4-nitro-4′-hydroxydiphenyl disulfide; 4-acetoxy-4′-benzoxydiphenyl disulfide; 2-nitro-4′-benzoxydiphenyl disulfide; 4-nitro-4′-benzoxydiphenyl disulfide; 4-benzoxyphenyl-2-anthraquinone disulfide; and 2-nitro-4′acetoxydiphenyl disulfide.

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Studies in Chemotherapy. V. The Synthesis of Certain Unsymmetrical Disulfide Derivatives of the 4-Nitrophenyl and the 4-Aminophenyl Radical*,†

BY IVAR DANIELSSON,‡ JOHN E. CHRISTIAN, and GLENN L. JENKINS

SINCE both symmetrical diaryl disulfides (1–4) and compounds where one-half of the symmetrical molecule has been substituted by an indifferent structure (4) have proved to be of value as chemotherapeutic agents, it was thought that unsymmetrical diaryl disulfides might possibly possess similar properties and with this thought in mind several compounds of the latter type have been synthesized.

According to the following reactions 4-nitrodiphenyl disulfide was synthesized. An alcoholic solution of 4-chloronitrobenzene was refluxed with sodium disulfide yielding bis(4-nitrophenyl) disulfide. The disulfide was purified by reducing it to the corresponding thio and oxidizing this product to the disulfide again. By the action of chlorine under anhydrous conditions on bis(4-nitrophenyl) disulfide suspended in chloroform, 4-nitrophenylsulfenyl chloride was obtained. The interaction of 4-nitrophenylsulfenyl chloride and benzenethiol resulted in the desired product, 4-nitrodiphenyl disulfide. The compound was also prepared from sodium benzenethiolate and 4-nitrophenylsulfenyl chloride. The dry sodium salt used was obtained by refluxing sodium and benzenethiol in benzene and evaporating the solvent when the reaction had gone to completion. The action of 4-methylbenzenethiol and 2-methylbenzenethiol on 4-nitrophenylsulfenyl chloride resulted in 4-methyl-4′-nitro-, and 2-methyl-4′-nitrodiphenyl disulfides, respectively.

An attempted synthesis of bis(4-aminophenyl) disulfide from aniline, lead carbonate, and sulfur failed, a gelatinous, uncrystallizable compound being obtained instead of the desired product.

The compound was, however, successfully synthesized by the reduction of bis(4-nitrophenol) disulfide by stannous chloride and hydrochloric acid and subsequent oxidation of the resulting 4-aminobenzenethiol to the disulfide. Acetylation of bis(4-aminophenyl) disulfide yielded bis(4-acetamidophenyl) disulfide. From the latter 4-acetamido-3-chlorophenylsulfenyl chloride was made by chlorination. By reacting 4-acetamido-3-chlorophenylsulfenyl chloride with 4-methyl-, 3-methyl-, and 2-methylbenzenethiols, the compounds 4-acetamido-3-chloro-3′-methyl-, and 4-acetamido-3-chloro-2′-methyldiphenyl disulfides, respectively, were synthesized.

By the following reactions 2,4-dinitro-4′-methyldiphenyl disulfide was prepared. An alcoholic solution of 2,4-dinitrochlorobenzene was refluxed with sodium disulfide yielding bis(2,4-dinitrophenyl) disulfide which when suspended in nitrobenzene and treated with chlorine gave 2,4-dinitrophenylsulfenyl chloride. The latter when reacted with 4-methylbenzenethiol gave the desired disulfide. When 2,4-dinitrophenylsulfenyl chloride was reacted with benzenethiol, 3-methyl-, and 2-methylbenzenethiols the compounds 2,4-dinitro-, 2,4-dinitro-3′-methyl-, and 2,4-dinitro-2′-methyldiphenyl disulfides, respectively, were obtained.

From 2-chloronitrobenzene and sodium disulfide bis(2-nitrophenyl) disulfide was prepared and the latter chlorinated, resulting in 2-nitrophenylsulfenyl chloride. The chloride was reacted with benzenethiol, 4-methyl-, 3-methyl-, and 2-methylbenzenethiols yielding 2-nitro-, 4-methyl-2′-nitro-, 3-methyl-2′-nitro-, and 2-methyl-2′-nitrodiphenyl disulfides, respectively.

* For previous papers in this series see THIS JOURNAL, **35**, 328–35(1946); **36**, 257–60(1947).

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‡ Eli Lilly and Company Research Fellow

EXPERIMENTAL

1. Bis(4-Nitrophenyl) Disulfide.—The procedures used were those of Wohlfahrt (5) and Bogert and Stull (6). The compound was purified according to the method described by Zincke and Lenhardt (7); m. p. 181°.

2. 4-Nitrophenylsulfenyl Chloride.—The procedure used was a modification of the one described by Hubacher (8). A current of chlorine was bubbled through 1.54 Gm. of bis(4-nitrophenyl) disulfide suspended in 20 cc. of chloroform. In this experiment as well as in all others where arylsulfenyl chlorides are either being prepared or used in reactions, conditions must be anhydrous in order to avoid undesired by-products. The reaction was completed in twenty minutes resulting in a clear solution of 4-nitrophenylsulfenyl chloride (0.01 mole). Excess chlorine contained in the system was expelled by passing a current of dry nitrogen through the reaction mixture. The solution was used without isolating the compound because of the extreme instability of the latter.

3. 4-Nitrodiphenyl Disulfide.—To a chloroform solution containing 1.9 Gm. (0.01 mole) of 4-nitrophenylsulfenyl chloride was added slowly a solution of 1.10 Gm. (0.01 mole) of benzenethiol in dry chloroform and the reaction mixture was refluxed for two hours, copper-bronze being used as a catalyst. Then the chloroform was evaporated off and the residue was recrystallized from hexane; yellow needles, m. p. 58–58.5°; yield 1.80 Gm. (68.4%).

Anal.—Calcd. for $C_{12}H_9O_2NS_2$: S, 24.35%; N, 5.32%. Found: S, 24.51%; N, 5.27%.

4. 4-Methyl-4′-nitrodiphenyl Disulfide.—The procedure used was the same as that for 4-nitrodiphenyl disulfide. Starting materials were 1.9 Gm. (0.01 mole) of 4-nitrophenylsulfenyl chloride and 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol. Yellow needles, m. p. 62–62.5°; yield 2.65 Gm. (95.5%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%; N, 5.05%. Found: S, 23.09%; N, 5.13%.

5. 2-Methyl-4′-nitrodiphenyl Disulfide.—The procedure used was the same as that for 4-nitrodiphenyl disulfide. Starting materials were 1.9 Gm. (0.01 mole) of 4-nitrophenylsulfenyl chloride and 1.24 Gm. (0.01 mole) of 2-methylbenzenethiol. Yellow needles, m. p. 84–84.5°; yield 2.30 Gm. (82.9%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: S, 22.81%.

6. Attempted Synthesis of bis(4-Aminophenyl) Disulfide from Aniline, Lead Carbonate, and Sulfur.—The procedure described by Lefevre and Desgres (9) was followed. Their results could not be reproduced, a gelatinous, uncrystallizable compound being obtained instead of the desired product. Lefevre and Desgres reported the compound to melt at 106°.

7. Bis(4-Aminophenyl) Disulfide Obtained by Reduction of bis(4-Nitrophenyl) Disulfide.—The method used was a modification of the one described by Shukla (10). To a refluxing suspension of 15.4 Gm. (0.2 mole) of bis(4-nitrophenyl) disulfide in 30 cc. of glacial acetic acid was slowly added a solution of 101.5 Gm. of stannous chloride, $SnCl_2{\cdot}2H_2O$, in 120 cc. of concentrated hydrochloric acid and the reaction mixture was refluxed for one hour. On cooling a crystalline material precipitated which was separated and dissolved in water and sufficient 20% sodium hydroxide solution added to make the solution slightly alkaline. Hydrogen peroxide (30%) was then added, dropwise, to the alkaline solution whereby the desired product separated. Recrystallized from dilute alcohol it melted at 76–77°; yield 10.9 Gm. (87.7%). Shukla reports a m. p. of 85°; other melting points have also been reported (11, 12).

8. Bis(4-acetamidophenyl) Disulfide.—The procedure used was that of Khmel'Nitzkaya and Mikel's (11). Starting from 24.8 Gm. (0.1 mole) of bis(4-aminophenyl) disulfide the yield was 34 Gm. (97.7%); m. p. 215–217°; other melting points have also been reported (11, 13).

9. 4-Acetamido-3-chlorophenylsulfenyl Chloride.—A current of chlorine was passed through a refluxing suspension of 1.66 Gm. (0.02 mole) of bis(4-acetamidophenyl) disulfide in 25 cc. of chloroform. The reaction was completed in fifteen to twenty minutes, resulting in a clear solution of the desired compound (0.01 mole). Excess chlorine was expelled by passing a current of nitrogen through the solution. The solution was used as such without isolating the compound.

10. 4-Acetamido-3-chloro-4′-methyldiphenyl Disulfide.—A chloroform solution of 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol was slowly added to 2.36 Gm. (0.01 mole) of 4-acetamido-3-chlorophenylsulfenyl chloride dissolved in 25 cc. of chloroform. Copper-bronze was used as a catalyst. After one hour refluxing the chloroform was evaporated and the residue extracted several times with boiling heptane. The extracts were combined and chilled; the crystals formed were recrystallized from heptane. A white microcrystalline substance resulted, m. p. 113–114°; yield 1.6 Gm. (49.4%).

Anal.—Calcd. for $C_{15}H_{14}OClNS_2$: S, 19.70%. Found: S, 19.14%.

11. 4-Acetamido-3-chloro-3′-methyldiphenyl Disulfide.—The procedure used was that described in Experiment 10, the reactants being 0.01 mole each of 3-methylbenzenethiol and 4-acetamido-3-chlorophenylsulfenyl chloride. A microcrystalline, white compound resulted, m. p. 93.5–94.5°; yield 0.50 Gm. (15.4%).

Anal.—Calcd. for $C_{15}H_{14}OClNS_2$: S, 19.70%. Found: S, 19.22%.

12. 4-Acetamido-3-chloro-2′-methyldiphenyl Disulfide.—The procedure used was that described in Experiment 10, 2-methylbenzenethiol being used instead of 4-methylbenzenethiol. A white, microcrystalline compound resulted, m. p. 99–100°; yield 0.50 Gm. (15.4%).

Anal.—Calcd. for $C_{15}H_{14}OClNS_2$: S, 19.70%. Found: S, 19.02%.

13. Bis(2,4-Dinitrophenyl) Disulfide.—The procedure used was that of Fromm, *et al.* (14). Starting from 0.1 mole of 2,4-dinitrochlorobenzene a yield of 14 Gm. (71.4%) was obtained. Yellow needles from nitrobenzene decomposed without melting around 280°. Fromm reported the compound to explode at 280°.

14. 2,4-Dinitrophenylsulfenyl Chloride.—The procedure used was a modification of that described by Hubacher (8). A current of chlorine was bubbled through a hot (120–130°) suspension of 1.99 Gm. (0.02 mole) of bis(2,4-dinitrophenyl) disulfide in 25 cc. of nitrobenzene. The reaction was completed in one and one-half hours, resulting in a clear solution of the desired product (0.01 mole). Excess of chlorine was expelled by passing a current of nitrogen through the reaction mixture. This solution was used for the 4 following experiments. The compound may be isolated by evaporation of the solvent *in vacuo* and recrystallization of the residue from carbon tetrachloride; m. p. 94–95°.

15. 2,4-Dinitro-4′-methyldiphenyl Disulfide.—A solution of 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol in 15 cc. of nitrobenzene was added slowly to a solution of 2.35 Gm. (0.01 mole) of 2,4-dinitrophenylsulfenyl chloride in 25 cc. of nitrobenzene, and the reaction mixture was heated on a steam bath for one hour. Both solutions were heated to 100° before they were mixed together. Then the solution was transferred to an evaporating dish and the nitrobenzene was evaporated. The residue was recrystallized from alcohol. Yellow needles, m. p. 114–115°; yield 2.5 Gm. (77.5%).

Anal.—Calcd. for $C_{13}H_{10}O_4N_2S_2$: S, 18.89%. Found: S, 19.27%.

16. 2,4-Dinitro-3′-methyldiphenyl Disulfide.—The procedure used was the same as that for 2,4-dinitro-4′-methyldiphenyl disulfide except that 3-methylbenzenethiol was used instead of 4-methylbenzenethiol. Yellow needles, m. p. 74.5–75.5°; yield 1.9 Gm. (58.9%).

Anal.—Calcd. for $C_{13}H_{10}O_4N_2S_2$: S, 18.89%. Found: S, 18.72%.

17. 2,4-Dinitro-2 -methyldiphenyl Disulfide.—The procedure used was that described in Experiment 15 using 2-methylbenzenethiol instead of 4-methylbenzenethiol. Yellow needles, m. p. 100–100.5°; yield 2.0 Gm. (62.4%).

Anal.—Calcd. for $C_{13}H_{10}O_4N_2S_2$: S, 18.89%. Found: S, 18.53%.

18. 2,4-Dinitrodiphenyl Disulfide.—The procedure used was that described in Experiment 15, benzenethiol being used instead of 4-methylbenzenethiol. Yellow needles, m. p. 86–87°; yield 2.5 Gm. (81.8%).

Anal.—Calcd. for $C_{12}H_8O_4N_2S_2$: S, 20.80%. Found: S, 20.72%.

19. Bis(2-nitrophenyl) Disulfide.—The procedure used was that described by Bogert and Stull (6). Yellow needles from glacial acetic acid, m. p. 197–198°. Bogert and Stull reported 192–195°; other melting points have also been reported.

20. 2-Nitrophenylsulfenyl Chloride.—A modification of the procedure described by Hubacher (8), and the same one as that for 4-nitrophenylsulfenyl chloride, was used except that during the chlorination the reaction mixture was refluxed instead of applying no heat. This solution was used without isolating the compound for the following experiments.

21. 4-Methyl-2′-nitrodiphenyl Disulfide.—A solution of 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol in 20 cc. of chloroform was slowly added to a chloroform solution of 1.9 Gm. (0.01 mole) of 2-nitrophenylsulfenyl chloride, and the reaction mixture was refluxed for one hour. The solution was then transferred to a beaker and the chloroform was evaporated. The residue was recrystallized from methyl alcohol. Yellow needles, m. p. 73.5–74°; yield 2.13 Gm. (76.3%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: S, 22.55%.

22. 3-Methyl-2′-nitrodiphenyl Disulfide.—The procedure used was that described in Experiment 21, 3-methylbenzenethiol being used intead of 4-methylbenzenethiol. Yellow needles, m. p. 74.5–75.5°; yield 2.32 Gm. (83.7%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: 22.60%.

23. 2-Methyl-2′-nitrodiphenyl Disulfide.—The procedure described in Experiment 21 was followed except that 2-methylbenzenethiol was used instead of 4-methylbenzenethiol. Yellow needles, m. p. 103–103.5°; yield 2.32 Gm. (83.7%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: S, 22.88%.

24. 2-Nitrodiphenyl Disulfide.—The procedure used was that described in Experiment 21 except that benzenethiol was used instead of 4-methylbenzenethiol. Yellow needles, m. p. 49–50°; yield 2.07 Gm. (78.6%).

Anal.—Calcd. for $C_{12}H_9O_2NS_2$: S, 24.35%. Found: S, 23.82%.

SUMMARY

1. A method for synthesizing certain unsymmetrical diaryl disulfides has been described.

2. The following unsymmetrical diaryl disulfides have been prepared: 4-Nitrodiphenyl disulfide; 4-Methyl-4′-nitrodiphenyl disulfide; 2-Methyl-4′-nitrodiphenyl disulfide; 4-Acetamido-3-chloro-4′-methyldiphenyl disulfide; 4-Acetamido-3-chloro-3′-methyldiphenyl disulfide; 4-Acetamido-3-chloro-2′-methyldiphenyl disulfide; 2,4-Dinitro-4′-methyldiphenyl disulfide; 2,4-Dinitro-3′-methyldiphenyl disulfide; 2,4-Dinitro-2′-methyldiphenyl disulfide; 2,4-Dinitrodiphenyl disulfide; 4-Methyl-2′-nitro-

diphenyl disulfide; 3-Methyl-2′-nitrodiphenyl disulfide; 2-Methyl-2′-nitrodiphenyl disulfide; and 2-Nitrodiphenyl disulfide.

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The Determination of Camphor in Solution with Phenol in a High Percentage of Light Mineral Oil*

By ERIC D. ROBINSON and S. C. WERCH

The standard hydrazone method for the determination of camphor cannot be employed directly on a solution of camphor with phenol in a high percentage of mineral oil. When a large percentage of mineral oil is present, it adheres to the hydrazone crystals and interferes with the gravimetric analysis. A method is here presented which eliminates this difficulty. This method is based on the extraction of camphor with alcohol. A weighed sample is centrifuged after first being emulsified by the addition of 95% ethyl alcohol. This procedure gives recoveries averaging 99.6%.

WHEN CAMPHOR is in an alcoholic solution such as Spirits of Camphor or in a small amount of mineral oil as in the National Formulary preparation of Camphorated Phenol (1), the standard hydrazone method for the determination of camphor can be employed directly. Direct application of this method, however, does not give accurate results for camphor when this stearoptene is associated with phenol in a large quantity of mineral oil. This is the case in many of the "Antiseptic Oils" that are available on the market. When a large quantity of mineral oil is present, it adheres to the hydrazone crystals and interferes with the gravimetric analysis.

Because our laboratory has had the task of determining camphor in a number of preparations containing camphor and phenol in a high percentage of mineral oil, it has been found necessary to devise a method for the separation of camphor from the oily base. This has been accomplished by extracting the camphor with alcohol. A weighed sample is centrifuged after first being emulsified by the addition of 95% ethyl alcohol. This procedure gives excellent recoveries.

* Received Feb. 11, 1947, from the Medical Research Division, Plough, Inc., Memphis, Tenn.

METHOD

If a large percentage of mineral oil is present, proceed as follows: A 0.3- to 1.5-Gm. sample containing camphor, phenol, and mineral oil is weighed into a 15-ml. centrifuge tube. To this solution 1–3 ml. of 95% ethyl alcohol is added and the mixture shaken vigorously until an opaque emulsion is formed. The alcohol-camphor layer which forms is then separated from the mineral oil by centrifuging at about 3500 r. p. m. for at least three minutes. This separation procedure is best repeated about four times. The alcohol-camphor layer, after each extraction, is carefully poured into a 250-ml. volumetric flask. If a tiny drop of oil when warmed fails to give an odor of camphor, one can be reasonably

certain that all the camphor has been extracted along with the phenol. The extract is now made to volume and gently mixed. Any oil-droplets that may appear at this time will settle to the bottom of the flask.

From the center of the flask draw four 25-ml. aliquots of the alcohol-camphor solution by means of a pipette. Empty each aliquot into a 500-ml. Erlenmeyer flask, then proceed with the assay for camphor as in N. F. VIII for Camphor Spirit. The amount of camphor is calculated from the weight of camphor 2,4-dinitrophenylhydrazone.

If "gunk" should appear floating about the flask prior to filtering the hydrazone after it is formed, it indicates that too much alcohol had been employed in the above extraction and that some of the mineral oil got into solution. This mineral oil also must be removed. This is done by decanting off the floating material and dissolving in boiling alcohol. The globules of mineral oil will settle to the bottom. Next pour off the supernatant liquid and cool in an ice bath. Then wash what hydrazine crystals might have formed into the crucible and proceed as before to wash with water and to dry to constant weight.

When only a small percentage of mineral oil is present, no emulsion will result from the addition of alcohol and separation by centrifuging will not be necessary.

TABLE I.—RECOVERY OF CAMPHOR FROM STOCK SOLUTION NO. 1

Sample	Sample,[a] Gm.	Camphor Added, Gm.	Camphor Recovered, Gm.	Recovery, %
1	0.1883	0.0282	0.0283	100.4
2	0.0385	0.0058	0.0057	98.3
3	0.0385	0.0058	0.0058	100.0
4	0.3746	0.0562	0.0559	99.5
5	1.2060	0.1809	0.1805	99.8
6	0.9910	0.1487	0.1484	99.8
			Av.	99.6

[a] Samples 1–6 are amounts of 6 different weighed samples.

EXPERIMENTAL

Recovery experiments were carried out on two stock solutions. One (No. 1), containing a large percentage of mineral oil and duplicating the general formula of many of the "Antiseptic Oils" on the market, was made up with 5% phenol, 15% camphor, and 80% light mineral oil. The other (No. 2) included a small percentage of light mineral oil and duplicated the National Formulary preparation of Camphorated Phenol (30% phenol, 60% camphor, and 10% light mineral oil). Extraction of the camphor was carried out on Stock Solution No. 1, while direct application of the hydrazone method was applied to Stock Solution No. 2.

The results of the recovery tests are shown in Tables I and II. Recoveries from Stock Solution No. 1 averaged 99.6% and were included in Table I. Table II presents the findings obtained from Stock Solution No. 2. Here the recoveries averaged 98.9%.

TABLE II.—RECOVERY OF CAMPHOR FROM STOCK SOLUTION NO. 2

Sample	Sample,[a] Gm.	Camphor Added, Gm.	Camphor Recovered, Gm.	Recovery, %
1	0.1543	0.0926	0.0913	98.6
2	0.1543	0.0926	0.0914	98.7
3	0.1543	0.0926	0.0911	98.4
4	0.1543	0.0926	0.0916	98.9
5	0.1543	0.0926	0.0919	99.3
6	0.1543	0.0926	0.0920	99.4
				Av. 98.9

[a] Samples 1–6 are amounts in 25-ml. aliquots from 1.5430 Gm. in 250 ml. of alcoholic solution at 20°.

DISCUSSION

The addition of alcohol to Stock Solution No. 2, which duplicates the National Formulary preparation of Camphorated Phenol, does not produce an emulsion, thus permitting the direct application of the hydrazone method. The mineral oil present in the sample goes readily into alcoholic solution with the phenol and camphor. This is due to the presence of only a small amount of mineral oil. In any case, no significant amount of mineral oil contaminates the hydrazone crystals.

When the hydrazone method for the determination of camphor was applied directly to solutions of camphor and phenol in a high percentage of light mineral oil, as in Stock Solution No. 1, results were about five times greater than the actual amount of camphor present. In these cases, mineral oil to a considerable degree is adhered to the hydrazone crystals, thus interfering with the gravimetric evaluation. Alcoholic emulsification and subsequent separation of the camphor and alcohol by centrifuging eliminated this interference.

CONCLUSION

A method is here presented which gives excellent recoveries of camphor when it is in solution with phenol in a high percentage of mineral oil.

Under such circumstances it is necessary to extract the camphor with alcohol before it is possible to apply the hydrazone reaction.

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Influence of Bile Salts on the Absorption of Quinine*

By U. P. BASU, S. MUKHERJEE, and R. P. BANERJEE

An explanation is offered of the mechanism of absorption of quinine from the intestine on the basis of its interaction with bile salts. Quinine reacts with an excess of sodium dehydrocholate or tauroglycocholate in alkaline medium to form a colloidal solution. When the latter is subjected to dialysis through cellophane, previously swollen with zinc chloride, both quinine and the bile acids are found to diffuse out through the membrane. Quinine dehydrocholate is only partially peptized by an excess of bile salt solution, and the resulting colloid, on dialysis, behaves similarly.

IT IS KNOWN that in order to maintain an adequate concentration of quinine in the blood for a sufficient length of time, oral administration of the drug is preferred to parenteral, and a daily dose of 2 Gm. (30 gr.) is usually required. But, on the average, about one-third of this quantity passes through the circulatory system and is subsequently excreted almost entirely through the kidneys. No difference is noticed in the quantity of quinine excreted, whether the alkaloid is administered in the form of a highly soluble salt (e.g., the dihydrochloride) or as a sparingly soluble salt (e.g., the sulfate) (1). The major portion of the quinine ingested is considered by some to be removed by the reticulo-endothelial cells (2) to the liver and destroyed during the metabolic processes (3, 4). The possibility that a part of the ingested quinine is lost through nonabsorption in the alimentary tract also cannot be excluded. It would be of interest to undertake an investigation to find, if possible, means by which the above fate of quinine in the system may be altered.

Kelsey and Oldham have shown (5) that destruction of quinine is catalyzed by an oxidase present in the system. The observations of Anderson, Cornatzer, and Andrews (3) that removal of the liver helps in the urinary excretion of quinine tends to indicate that the liver is the main seat of the above oxidase, and of the consequent destruction of quinine. It may be noted that, in the distribution of quinine in the different organs of the body, the highest concentration is found in the liver, as determined by Ramsden, Lipkin, and Whitley (6). The question suggests itself whether, by increasing the flow of blood in the hepatic arteries, the concentration and/or retention of quinine in the liver and, therefore, its fate in the system might be altered. It is known that bile salts stimulate the hepatic flow (7) and also have the property of keeping in solution several types of water-insoluble substances. If they exert a similar action on quinine, the ingestion of one or other bile salt along with quinine might be of considerable significance.

The administration of quinine and other cinchona alkaloids in combination with bile acids has previously been claimed in the patent literature to enhance the parasiticidal action of the drugs (8–12), but the manner in which this action is exerted by the bile acids has not been explained. Such work on the influence of a hydrochloretic on the metabolism of quinine was considered to be of importance.

In the present study, the interaction of quinine with bile acids or salts has been investigated under various conditions. Salts of tauroglycocholic acid and dehydrocholic acid have been used for these investigations, since the former is a common constituent of bile, and the latter, an oxidation product of cholic acid, is well known for its marked effect on bile secretion (13) and low toxicity (14); [*cf.* also Gilbert (15)].

EXPERIMENTAL

Preparation of Quinine Dehydrocholate.—Quinine (16.4 Gm.) and pure dehydrocholic acid (20.1 Gm.) were dissolved separately in chloroform by warming. The respective solutions were filtered and mixed with stirring. The chloroform was then evaporated on a steam bath and the residual mass triturated with absolute alcohol and finally mixed with ether. The ether was removed and the resulting powder was then refluxed with dilute alcohol

* Received Jan. 29, 1947, from the Bengal Immunity Research Laboratory, Calcutta, India.

(1:1) for about one hour (with the addition of charcoal). The suspension was then filtered and the filtrate cooled. Quinine dehydrocholate separated out and was collected. It melted at 148–150° with decomposition. The melting point was not depressed when the salt was melted in a sealed tube.

Anal.—Calcd. for quinine, 44.6%; found: 43.9%.

Solubility of Quinine Dehydrocholate.—For the determination of solubility, 100 ml. of water was saturated at 28° with the salt, the filtered solution was evaporated and the residue dried at 100° to constant weight. The solubility of quinine dehydrocholate in water at 28° was found to be 0.0964 Gm. per 100 ml. of water.

Interactions of Quinine Salts with Sodium Dehydrocholate and Tauroglycocholate.—The following experiments were performed in order to study the reaction, and the nature of the products formed:

(A) To 7 ml. of a 0.0568 *M* quinine dihydrochloride solution (*p*H = 3.14) was gradually added a 0.025 *M* solution of sodium dehydrocholate (*p*H = 9.06) until the *p*H of the resulting mixture was 8.2. The volume of sodium dehydrocholate required was 11.75 ml. A coarse precipitate was formed. A similar precipitate was formed when a quinine hydrocholoride solution was used in place of the dihydrochloride.

(B) To 50 ml. of 0.0468 *M* sodium dehydrocholate was added dropwise 8.5 ml. of 0.068 *M* quinine hydrochloride; the *p*H of the resulting mixture was 8.4. The solution remained clear initially, but turbidity appeared as addition proceeded, and a colloidal solution was ultimately formed. The turbidity was measured by means of a Klett–Summerson photoelectric colori neter (the zero of which was set for water) and a blue filter (see Table I).

(C) An experiment was carried out as in B but using sodium tauroglycocholate in place of sodium dehydrocholate.

The same concentration (0.0468 *M*) of sodium dehydrocholate and tauroglycocholate was used in experiments B and C, but while the former initially gave a zero reading in the photoelectric colorimeter, the latter solution gave a reading of 104.

Examination of the reaction products showed that, while the colloid formed with sodium dehydrocholate was practically stable toward centrifuging, an appreciable quantity of a coarse sediment was thrown down from that obtained with sodium tauroglycocholate. When quinine dihydrochloride, which is the more acidic, was used instead of quinine monohydrochloride, coarse precipitates began to be formed as soon as the mixture became acidic; this could be prevented by intermittent addition of dilute caustic soda to maintain the reaction alkaline. But, even when the *p*H was controlled in this manner, any further addition of quinine after the molar ratio of quinine to the bile acid in the reaction mixture was about 1:4, resulted in the formation of coarse precipitate.

(D) A 0.0468 *M* quinine monohydrochloride solution was added gradually to a dilute solution of caustic soda until the *p*H of the final mixture was between 8.0 and 9.0. Only a coarse precipitate of the alkaloid was formed.

(E) Equivalent amounts of quinine base and dehydrocholic acid were suspended in water, the *p*H of the solution raised to 8.4 by the gradual addition of caustic soda, and the mixture shaken thoroughly. There was no observable colloid formation.

(F) The reaction mixtures obtained in experiments A, B, C, D, and E were centrifuged for one hour and the supernatant liquids tested for turbidity in the photoelectric colorimeter (Table I).

The colloidal solutions from experiments B and C were then subjected to dialysis in bags of cellophane previously swollen in 64% zinc chloride solution for twenty minutes. The dialysis was carried out against distilled water; the *p*H of the water was subsequently maintained at about 8.4 by the addition of the requisite quantity of caustic soda. Quinine, as well as the bile acid, was found to be diffusing out into the dialysate. The dialysis was continued, with daily changes of water, until no further diffusion of quinine into the outer liquid was noticeable. At this stage, the turbidity readings of the dialyzed liquids were again taken in the photoelectric colorimeter (Table I).

TABLE I

Experiment	Turbidity Reading of Centrifuged Mixture	Turbidity Reading after Exhaustive Dialysis
A	34	Not dialyzed
B	320	2 (did not give the test for quinine)
C	640	108 (there was coarse sediment containing quinine)
D	0	Not dialyzed
E	0	Not dialyzed

The first dialysate was somewhat turbid but subsequent dialysates were clear.

(G) Quinine dehydrocholate was finely powdered, suspended in water, made alkaline with caustic soda and shaken vigorously for fifteen minutes. There was practically no peptization of the solid.

(H) One-tenth gram of quinine dehydrocholate was finely powdered and shaken with 25 ml. of 0.0468 *M* sodium dehydrocholate solution for half an hour and then centrifuged for one hour. A part of the solid was peptized to give a stable colloidal solution. Analysis of this supernatant liquid showed that nearly 10% of the quinine dehydrocholate taken was peptized in this manner.

Use of sodium tauroglycocholate in place of sodium dehydrocholate also resulted in partial peptization of the quinine salt.

(I) The peptized colloidal solution of quinine dehydrocholate solution was also subjected to exhaustive dialysis. A slight turbidity persisted in the liquid even after ten days' dialysis, although tests showed that most of the quinine taken had passed out into the dialysate. Most of the dehydro-

cholic acid was also found to have diffused out through the membrane.

DISCUSSION

It will be seen from the results of the experiments recorded above that quinine readily forms a salt with dehydrocholic acid in chloroform solution, but this salt is sparingly soluble in water and requires more than 1000 parts of water to dissolve it; but when the interaction between a quinine salt and sodium dehydrocholate or tauroglycocholate takes place in aqueous medium, the nature of the product of reaction is found to vary with the conditions of the experiment.

When the quinine salt was added to the bile salt, both being in aqueous solution, and the reaction of the medium was maintained at a *p*H between 8.0 and 9.0 during the addition, the mixture gradually developed turbidity and took the form of a stable colloidal solution (Experiments B and C). The reverse mode of addition, on the other hand, produced a coarse precipitate (Experiment A). It was demonstrated that quinine is precipitated from solution at the *p*H and concentration of the above experiments (Experiment D). Treatment of a mixture of quinine base and dehydrocholic acid (Experiment E) or of quinine dehydrocholate (Experiment G) with dilute caustic soda solution produced no observable effect. Quinine dehydrocholate was, on the other hand, found to be peptized by excess of an alkaline solution of sodium dehydrocholate (Experiment H), although the proportion of the solid peptized in this manner was found to be about 10%.

Dialysis Experiments F and I on the colloids obtained from Experiments B, C, and H show that, in all these cases, the quinine as well as the bile acid diffused out through the cellophane membrane, to a greater or less extent. The diffusion of quinine was complete in the case of the colloid obtained from Experiment B but incomplete with the colloids from Experiments C and H. It thus appears that bile salts, under favorable conditions, can "solubilize" quinine in an alkaline medium.

Quinine, when taken orally, is considered to be absorbed mainly from the intestines (16). Bile is also normally present in this region. When *ingested quinine* enters the intestine in the form of a solution and reacts with the bile salts, the result, presumably, would be a colloidal solution as in Experiment C. Since quinine is found to diffuse through swelled cellophane membrane from this type of colloid, it might also be expected to penetrate the membranes of the cells lining the stomach and thus enter the circulatory system.

It will be seen from Experiment C that a considerable excess of bile salts would be necessary in order to have the quinine in the form of a stable colloidal solution. It would therefore follow that the presence of an adequate quantity of bile salts would be necessary for the efficient absorption of quinine from the intestine. Recent work by Fredericks and Hoffbauer (17) has shown that attacks of therapeutic malaria are almost always accompanied by hepatic disturbances; these may disturb the flow of the normal quantity of bile from the liver into the intestine. In the treatment of malaria by the oral administration of quinine, therefore, the simultaneous administration of an effective choleretic like dehydrocholic acid is likely to help in the absorption of quinine and thereby to enhance its therapeutic effect, particularly in those cases where deficiency of bile secretion is suspected.

Further, Experiment H indicates that the administration of quinine in the form of salts of bile acids is not likely to be of any special advantage. Such a salt of quinine would be peptized or solubilized by bile salts only partially, the major part remaining as an insoluble precipitate, the absorption of which would not be very efficient.

SUMMARY

1. Quinine and dehydrocholic acid react in chloroform solution to form a salt containing one molecule of each component. This salt is sparingly soluble in water.

2. When added to an alkaline bile salt or dehydrocholate solution, a quinine salt in aqueous solution forms a colloidal solution. On dialysis of this colloidal solution, both quinine *and* the bile acids diffuse out through the cellophane membrane.

It has been suggested that this solubilizing action of bile salts on quinine in alkaline medium provides a mechanism for the absorption of quinine from the intestine.

3. The simultaneous administration of an effective choleretic, preferably dehydrocholic acid, in the quinine therapy of malaria might augment the therapeutic efficacy of the drug.

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A Spectrophotometric Method of Analysis of Cascara Sagrada Preparations*

By MELVIN R. GIBSON and ARTHUR E. SCHWARTING

A method of quantitative analysis of Cascara Sagrada preparations dependent upon the isolation of the hydroxy-methyl-anthraquinones on a chromatographic column of magnesium oxide and celite was devised. The anthraquinones were quantitatively eluted from the column and the concentration of the resulting solutions determined from the results of spectrophotometric examinations. Calculations of concentration of total anthraquinones were made on the basis of a determined extinction coefficient for pure emodin. The results of the analyses of fluidextracts by this method gave results correlating quite closely those previously reported in the literature for a different process.

THE PROBLEM of obtaining an accurate means of quantitative analysis for the anthraquinone-bearing drugs has been under investigation for over a half century. The methods which have received attention during this time include (*a*) gravimetric extraction, (*b*) gravimetric precipitation, and (*c*) colorimetric. Gravimetric extractions have been studied by Aweng (1), Daels (2), Tunmann (3), Fuller (4), and Hebeisen (5). Gravimetric precipitation methods have been investigated by Tschirch and Edner (6), and Tschirch and Pool (7). Colorimetric methods have been suggested by Tschirch and Heipe (8), Tschirch (9), Bromberger (10), Warin (11), Maurin (12), and Fuller (13).

In 1926 Tumminkatti and Beal (14) proposed a method of analysis involving Daels' method with modifications to include quantitative adsorption of the anthraquinones on Norit decolorizing carbon. This process involved the weighing of the impure extracted anthraquinones before and after treatment with Norit and calculating the anthraquinone quantity to be the difference.

The results of this method of assay seemed to the authors to be indicative that this method was the best that has been evolved in solving the problem of quantitative analysis of anthraquinone-bearing drugs. However, in the process of an extensive study of the chromatographic isolation and spectrophotometric analysis of the anthraquinone constituents of the bark of Cascara Sagrada being carried on by the authors, a simplification of this process, following generally the same line of reasoning, was believed possible.

One of the disadvantages of the process of Tumminkatti and Beal is the tedious shaking-out process in the isolation which yields large volumes of liquids that become difficult to handle and which must eventually be concentrated. To alleviate this difficulty as well as the weighings of minute quantities, chromatographic isolation and quantitative spectrophotometry are suggested.

Ernst and Weiner, as discussed by Zechmeister and Cholnoky (15), were the first to study extensively the anthraquinone-bearing drugs by means of chromatography. The resolution of an alcoholic extract into anthraquinone and anthranol layers was accomplished for ten anthraquinone-bearing drugs.

EXPERIMENTAL

In the method to follow, the isolation of the anthraquinones (the term *anthraquinones* is used in this paper to mean the trihydroxy-methyl anthraquinones of the emodin series) is dependent upon chromatography and the quantitative determination upon the adsorption of light as measured by the spectrophotometer. The method of extraction of anthraquinones present in the "free" or uncombined state and those present in the "combined" or as aglycones of glycosides was essentially that of Daels (2). For the analysis of the powdered extract and powdered bark of Cascara Sagrada, 5 Gm. was used and for the fluidextracts of Cascara Sagrada, 5 cc. was pipetted onto shredded filter paper and allowed to dry in a 56° constant temperature oven. The powder or dried shreds were then placed in a 500-cc. round bottom flask with 250 cc. of chloroform and refluxed for one hour on a steam bath. The

* Received February 13, 1947, from the Department of Pharmacognosy, College of Pharmacy, University of Nebraska, Lincoln, Nebr.

chloroform was decanted and the Cascara washed with two 10-cc. portions of chloroform. This chloroform solution contains the "free" anthraquinones. The partially extracted material was then refluxed in the same flask for two and one-half hours in the presence of 250 cc. of chloroform and 50 cc. of 25% sulfuric acid. The chloroform and aqueous mixture was decanted from the marc which was then washed with three 20-cc. portions of chloroform. The chloroform was separated from the aqueous layer in a separatory funnel. This chloroform solution contains the anthraquinones present in the preparations in glycosidal form which have been separated by hydrolysis. This solution will be referred to as the "combined" anthraquinones.

These solutions were then allowed to pass by continuous gravity-flow through chromatographic tubes packed with a mixture of Westvaco Adsorptive Magnesia #2641 (1 part) and celite (2 parts). The chloroform solution of free anthraquinones yielded a small bright red layer of anthraquinones at the top of the column followed by a yellow layer of anthranols which gradually became dispersed throughout the column. The anthraquinones remained in a definite red layer at the top. The solution passing through the column did not yield a Borntraeger Reaction (16) indicating the absence of anthraquinones. Sectioning of the extruded column proved that all anthraquinones were adsorbed in the top red layer. This would be expected since the basicity of the magnesia column causes the bright red layer to form wherever anthraquinones are present. The red layers formed by the chromatograms of free anthraquinones were 5 to 7 ml. wide and those for combined anthraquinones were from 12 to 24 ml. wide in standard chromatographic tubes 19 ml. in diameter.

The columns were extruded and the red layers cut from the remainder of the tube. Ordinary methods of eluting the adsorbed anthraquinones were unsuccessful. However, by treating the dry red powder with 10% hydrochloric acid the chemical reaction of the acid with magnesium oxide released the adsorbed anthraquinones which were readily shaken from the acid-aqueous mixture, in which they are insoluble, with successive portions of chloroform. For the free anthraquinone layer obtained from the fluidextracts, chloroform in 25-cc. portions was shaken with the mixture until the chloroform washings, measured in a volumetric flask, totaled 250 cc. For the powdered extract a total of 500 cc. of chloroform was shaken with the mixture and for the powdered bark a total of 1000 cc. was used. Tests of subsequent shakings beyond these volumes demonstrated an absence of anthraquinones. The procedure for the elution of the layers containing the combined anthraquinones of all samples was similar to the method used to elute the free anthraquinones. The chloroform washings, however, were continued until the solutions in each case measured 1000 cc. in a volumetric flask. Before spectrophotometric tests could be made 10 cc. of the chloroform solution representing the free anthraquinones of the powdered drug and the chloroform solutions of the combined anthraquinones of the powdered extract and the powdered drug were each diluted to 100 cc. quantitatively. All other solutions were examined as originally prepared.

These chloroform solutions were examined spectrophotometrically using a Coleman Universal spectrophotometer in the visible region of the spectrum at ten-millimicron intervals using the null method. The solutions were in cuvettes 1.3 cm. in diameter using chloroform as the standard. A complete analysis of the eluted free and combined chloroform solutions of the respective chromatograms of sample C-2, a powdered extract, is shown in Table I.

Pure emodin was prepared from Cascara Sagrada bark by the Jowett (17) method and a solution of this examined spectrophotometrically gave results as shown in Table I. Preliminary investigations of the other anthraquinones isolated from Cascara indicate maximum light absorption in the 400 to 440 mμ range. The maximum absorption of the red layers is the same as that for pure anthraquinones indicating anthraquinone isolation in the red layer of the chromatogram.

TABLE I.—COMPARATIVE TRANSMITTANCE OF ELUTED CHROMATOGRAPHIC LAYERS AND EMODIN

Wave Length, mμ	C-2 Free	C-2 Comb.	Emodin
400	54.5	66.0	69.5
410	57.0	68.5	67.0
420	58.0	69.0	64.0
430	58.0	69.5	60.5
440	58.5	70.0	59.0
450	60.5	71.5	59.5
460	64.5	74.3	63.0
470	71.5	79.0	68.7
480	79.0	84.5	76.5
490	86.0	88.5	83.0
500	91.0	93.0	88.5
510	94.0	94.0	91.5
520	96.5	96.5	94.0
530	97.5	98.0	94.0
540	98.0	98.0	95.0
550	99.5	98.5	96.0
560	99.5	98.5	96.0
570	99.5	98.5	96.5
580	99.5	99.0	96.5
590	99.5	99.0	96.5
600	99.5	99.0	97.0

Since emodin is the anthraquinone present in the largest quantity, all quantitative calculations are made on the emodin basis at the wave length of maximum absorption for emodin, 440 mμ. Since all anthraquinones show maximum or near-maximum absorption at this wave length and are simply position isomers of emodin the molecular extinction coefficient (K) for emodin is a workable basis.

Emodin was proved to conform with Beer's law in that the absorptance of the solution is directly proportional to the concentration of the solute (number of absorbing molecules of absorbing sub-

TABLE II.—PERCENTAGE TRANSMITTANCE AT 440 mμ

Sample	Free	Combined
A-1	74.0	36.0
A-2	76.0	35.3
A-3	35.3	45.0
B-1	58.0	17.0
B-2	72.0	17.0
B-3	45.0	17.0
C-1	60.0	69.0
C-2	58.5	70.0
C-3	64.5	68.3
D-1	77.0	53.0
D-2	77.0	52.0
D-3	77.0	52.7

TABLE III.—CALCULATED WEIGHTS (GM./5 CC. OR GM./5 GM.) OF ANTHRAQUINONES

Sample	Free	Combined	Total
A-1	0.0010	0.0135	0.0145
A-2	0.0009	0.0137	0.0146
A-3	0.0034	0.0105	0.0139
B-1	0.0017	0.0232	0.0249
B-2	0.0010	0.0232	0.0242
B-3	0.0026	0.0232	0.0258
C-1	0.0033	0.0490	0.0523
C-2	0.0035	0.0470	0.0505
C-3	0.0028	0.0503	0.0531
D-1	0.0317	0.0836	0.1153
D-2	0.0317	0.0869	0.1186
D-3	0.0317	0.0843	0.1160

stance). Three solutions of known concentration of emodin were prepared. Solution No. 1 contained 0.010 mg. per cc., No. 2, 0.005 mg. per cc., and No. 3, 0.0025 mg. per cc. At 440 mμ, No. 1 gave 47.0% transmittance, No. 2, 68.5%, and No. 3, 84.7%. When the transmittance is plotted on a logarithmic axis against concentration, a straight line results indicating conformity to Beer's law.

Using the formula $C = \frac{\log_{10} Io/I}{Kb}$ the emodin K is 6830. In the formula, C equals the concentration in moles per liter, $\log_{10} Io/I$ is the logarithm of the ratio of incident to emergent light, K is the molecular extinction coefficient, and b is the thickness of the absorption cell. The value of the molecular extinction coefficient for emodin was used in conjunction with this formula in subsequent calculations of the anthraquinone content of the Cascara preparations.

Using the processes of extraction, isolation, and quantitative measurement as indicated above, the following preparations were analyzed. Samples of the A series represent a U. S. P. Fluidextract of Cascara Sagrada made by a prominent manufacturer; samples of the B series represent a U. S. P. Fluidextract of Cascara Sagrada by a different manufacturer; samples of the C series represent the powdered U. S. P. Extract of Cascara Sagrada made by the same manufacturer as the Fluidextract A; and samples of the D series represent the bark of Cascara Sagrada in a powder finer than #40.

The percentage transmittance of the anthraquinone 440 mμ is given in Table II. Table III gives the calculated results in grams.

SUMMARY

From these results several conclusions may be drawn concerning the process.

1. As exemplified by samples A the results for free and combined anthraquinones vary only a few milligrams from the results reported by Tumminkatti and Beal in their process using an extract from the same manufacturer.

2. As shown by the results of sample B there is considerable difference in anthraquinone content of the products of different manufacturers. This was also found to be true by Tumminkatti and Beal.

3. The processes of extraction and isolation seem to be consistently efficient as several successive samples gave exactly the same spectrophotometric readings and all were within 1 or 2 mg.

4. The preparations of Cascara Sagrada represent only a fraction of the anthraquinones present in the bark.

An efficient method is proposed for the quantitative analysis of Cascara Sagrada preparations aimed at eliminating some of the factors influencing variable results of previous methods of analysis.

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The Preparation and Properties of the Perchlorates of Some Choline Esters*,†

By FREDERICK K. BELL and C. JELLEFF CARR

The preparation and properties of acetyl, benzoyl, furoyl, and thenoyl choline perchlorates are described. It is shown that perchloric acid may be used to isolate choline esters from their reaction mixtures.

THE PREPARATION of a number of choline esters which were desired for pharmacologic study in this laboratory was greatly facilitated by the observation that acetyl choline can be readily obtained in a well-defined crystalline form as the perchlorate salt. Furthermore, even casual observation revealed that this salt showed considerable stability toward atmospheric moisture in marked contrast to the well-known behavior of the chlorides of most of the choline esters that have been prepared. Our hope that the desired choline esters would also yield similar perchlorate salts was fulfilled.

A survey of the literature reveals that little attention has been given to the preparation of the perchlorate salts of choline esters. Hofmann and Hobold (1) have prepared and described in detail choline perchlorate and the nitrate ester of choline perchlorate. In accord with their recommendations, the latter compound has been found very satisfactory for the identification of choline (2) and its pharmacology has been investigated in this laboratory (3). Binet and Burstein (4) studied the action of propionylcholine perchlorate on the bronchomotor apparatus but give very little information concerning the chemistry of the salt.

It is the purpose of this communication to describe the preparation and properties of the perchlorates of four choline esters, *viz.*, acetyl, benzoyl, furoyl, and thenoyl. The latter two compounds are new esters that have not been previously prepared. It will be noted that if the sulfur atom in the thiophene ring of the thenoyl compound is replaced by oxygen then the furoyl ester is obtained.

EXPERIMENTAL

Acetyl Choline Perchlorate.—This salt can be readily obtained in the following manner and in good yield. To a 10% solution of acetyl choline chloride or bromide in absolute ethyl alcohol, add dropwise, with stirring, 60% perchloric acid solution until slightly more than the calculated amount of acid has been added. Chill the mixture in ice while stirring. The perchlorate is precipitated as a mass of feathery white crystals, which can be readily removed by filtration. If the crystals are washed with a little ice-cold absolute alcohol and then with dry ether, a pure product is obtained. After 2 recrystallizations from absolute alcohol the compound melted at 116–117°.[1] The crystals occur as white rods or needles, are double refracting, and are readily soluble in water, acetone, and hot absolute alcohol. Microanalysis in duplicate gave the following results:

Anal.—Calcd. for $C_7H_{16}O_6NCl$: C, 34.22%; H, 6.57%; N, 5.70%. Found: C, 34.45% and 34.32%; H, 6.90% and 6.82%; N, 5.58% and 5.59%.

The benzoyl, furoyl, and thenoyl esters of choline were readily prepared by condensing choline chloride with the corresponding acid chloride. Thenoyl chloride was prepared by treating α-thenoic acid (kindly supplied by the Socony-Vacuum Company) with thionyl chloride (5).

In the case of each of the 3 esters the procedure was similar. In a suitable flask equipped with a vertical air condenser, choline chloride was treated with the acid chloride in the proportion of 1 mole of the former to 2 or 3 moles of the latter. The mixture was heated on a steam bath for several hours. The mixture was cooled and then extracted several times with dry ether to remove the excess acid chloride and hydrochloric acid. The residue, consisting of the chloride of the ester, was dissolved readily in absolute alcohol. On treating this solution with a slight excess of 60% perchloric acid solution the perchlorate salt was precipitated and subsequently purified by recrystallization.

Each of the 3 salts appears to be completely nonhygroscopic. A report on the pharmacology of these compounds is in the course of preparation.

Benzoyl Choline Perchlorate.—White double refracting crystals, m. p. 203–205°; readily soluble in acetone, much less soluble in hot absolute alcohol, sparingly soluble in water.

* Received March 10, 1947, from the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md.

† The expense of this study was defrayed in part by a grant from the Board of Trustees of the United States Pharmacopœial Convention.

[1] All melting points reported were determined with the Fisher–Johns melting-point apparatus.

Anal.—Calcd. for $C_{12}H_{18}O_6NCl$: C, 46.83%; H, 5.90%; N, 4.55%. Found: C, 47.16% and 47.45%; H, 6.22% and 6.11%; N, 4.50% and 4.52%.

Furoyl Choline Perchlorate.—White double refracting crystals, m. p. 143–144°; readily soluble in acetone, soluble in water, and less soluble in absolute alcohol.

Anal.—Calcd. for $C_{10}H_{16}O_7NCl$: C, 40.34%; H, 5.42%; N, 4.71%. Found: C, 40.37% and 40.29%; H, 5.78% and 5.40%; N, 4.76% and 4.60%.

Thenoyl Choline Perchlorate.—White double refracting scales or plates; m. p. 189–190°; readily soluble in acetone, sparingly in alcohol and water.

Anal.—Calcd. for $C_{10}H_{16}O_6NHClS$: C, 38.28%; H, 5.14%; N, 4.46%. Found: C, 38.50% and 38.61%; H, 5.26% and 5.60%; N, 4.48% and 4.49%.

As has been previously noted, with the first preparation of acetyl choline perchlorate we were impressed by the apparent stability of this compound toward atmospheric moisture. This is in definite contrast to the behavior of the chloride which is usually dispensed in sealed glass ampuls because of its marked hygroscopicity. Acetyl choline bromide is regarded as more stable than the chloride in this respect. We have carried out several simplified experiments to demonstrate the comparative behavior of these 3 salts toward atmospheric moisture under controlled conditions.

Four similar weighing bottles were selected and each of 3 of these was weighed against the 4th bottle as a tare. Into 1 bottle was introduced approximately 0.1 Gm. of acetyl choline perchlorate which had been stored over calcium chloride. The bottle was immediately closed and then weighed. In the same manner a 0.1-Gm. sample of acetyl choline bromide was introduced into a second weighing bottle and weighed. This salt was removed from a new and unopened bottle of the Eastman Kodak product. The label was removed from a sealed glass ampul containing 0.1 Gm. of Merck's acetyl choline chloride and the ampul was then cleaned and dried. The ampul was opened and immediately placed in the third weighing bottle, which was promptly stoppered and then weighed.

A water-sulfuric acid mixture adjusted to give a relative humidity of 50% at a room temperature of 25° was introduced into a desiccator in place of the usual desiccant. The weighing bottles were placed in the desiccator and opened, after which the cover was placed on the desiccator. At definite intervals the bottles were stoppered, removed from the desiccator, and weighed, after which they were returned to the desiccator for further exposure to the humidified atmosphere. The weighing bottle used as a tare was subjected to the same treatment throughout the experiment.

A typical series of weighings is shown in Table I. These results indicate that the perchlorate is completely stable under the conditions of the experiment. On the other hand the chloride takes up moisture rapidly and the bromide shows a definite hygroscopicity which, however, is considerably less than that of the chloride.

In the light of these results we subjected the bromide and the perchlorate to a more drastic test. The sulfuric acid mixture in the desiccator was replaced with water and under these conditions a relative humidity of 100% was to be expected. Samples of a tenth of a gram of each of the 2 salts were weighed out and, after exposure to this atmosphere for one hour, were weighed again. The bromide had visibly liquefied and showed a weight increase of 10 mg. or 10%. No significant change in weight of the perchlorate sample was observed.

We may therefore conclude that the perchlorate is completely nonhygroscopic. It is readily soluble in water and hence represents a very convenient form of acetyl choline for the pharmacologist, especially in those cases where this substance must be weighed out in small quantities with analytical precision.

TABLE I

Total Time of Exposure, Hr.	Total Change in Weight of 0.1-Gm. Samples, Mg.		
	Chloride	Bromide	Perchlorate
1/4	1.7	0.7	−0.3
1/2	2.8	1.2	−0.2
1	3.9	1.5	−0.1
1 1/2	5.4	1.8	+0.1
2 1/2	7.8	2.4	−0.1
3 1/2	10.6	3.4	0.0
4 1/2	12.7	4.0	−0.1
10 1/2	20.6	6.1	+0.1
51 1/2	39.2	8.1	−0.2

SUMMARY

1. The preparation and properties of acetyl, benzoyl, furoyl, and thenoyl choline perchlorates have been described.

2. The results indicate that perchloric acid may be used to considerable advantage to isolate choline esters from their reaction mixtures since in general the perchlorate salts are well-defined crystalline and nonhygroscopic substances.

3. Acetyl choline perchlorate, which can be readily prepared, is nonhygroscopic and readily soluble in water. It therefore represents a highly desirable form of acetyl choline particularly for the pharmacologist's use.

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Organic Ozonides as Chemotherapeutic Agents. I. Chemical Studies*

By GEORG CRONHEIM†

Chemical and physical properties of ozonized olive oil have been investigated. Ozonized olive oil decomposes in the presence of water into medium length mono- and dibasic acids and aldehydes and hydrogen peroxide. Organic peroxidic compounds are formed as intermediates. It is suggested that the beneficial clinical results which have been obtained with ozonized olive oil are due to the germicidal properties of the peroxides and to specific actions of the final decomposition products.

THE PHARMACEUTICAL use of oxygen releasing substances dates back as far as 1818 when Thenard discovered hydrogen peroxide. The compounds employed since then were and still are almost exclusively inorganic or organic peroxides. Very little attention has been paid to ozonides many of which decompose with the release of nascent oxygen and can thus be used in the same manner as peroxides.

Although Schoenbein was the first to observe in 1855 the addition of ozone to the ethylene linkage, it was Harries who from 1901–1916 studied in detail the formation and the physical and chemical properties of organic ozonides. In 1914 Knox (1) filed a patent application for "a medicinal compound comprising the ozonide of the triglyceride of a hydroxylated fatty acid of the $C_nH_{2n-2}O_3$ series." This ricinoleic acid ozonide was supposed to be an "improved, efficient and emollient laxative" with germicidal action upon pathogenic microorganisms in the intestines.

As a matter of curiosity mention should also be made of the book *Experiments with Oxygen on Disease*, published in 1921 by James Todd (2). The author first deals in detail with the manufacture of ozonized olive and cod-liver oils. He then goes on to describe how he obtained "miraculous cures" with oral doses of "from 100 to 300 drops daily of the ozonized oil alone or with a small quantity of whisky" in Bright's disease, tuberculosis, etc.

A scientific investigation of the germicidal properties of ozonides in form of ozonized olive oil was started through the investigations of Harada (3) and Stevens (4). The first clinical report was published by Bender and Blanchard (5) in 1938, followed by reports from Sawyer (6), Sackin (7), Leonard and Engle (8), Greenberger and Helfert (9), Barrows (10), and recently by Sharlit (11).

In all these cases the ozonide was used topically. The only investigation on the internal use of ozonides seems to be that of Butz and La Lande (12) who studied the anthelmintic action of several ozonized oleic acid esters in dogs infested with ascarides.

The reason that ozonized olive oil has been used in these investigations is due to the fact that olive oil with its relatively high concentration of olefinic double bonds is a very convenient starting material. Furthermore, as will be shown later, it has certain advantages regarding stability and odor over other investigated materials and synthetic oleic acid esters.

Ozonized olive oil[1] is obtained by forcing an ozone-air mixture through olive oil at room temperature. Under proper conditions, the ozone will react quantitatively with the double bonds in the unsaturated fatty acids forming the corresponding ozonides. It has been found that a partly (50 per cent) ozonized olive oil represents the most practical form for pharmaceutical application and such a product was used for the most part in the present investigation.

The main components in olive oil with olefinic double bonds are oleic and linoleic acids. Calculated as triolein and trilinolein, they comprise from 75–85 per cent and from 6–10 per cent, respectively, of the olive oil,

* Received Feb. 10, 1947, from the Research Laboratories of The G. F. Harvey Company, Saratoga Springs, N. Y.

† Present address: The S. E. Massengill Company, Bristol, Tenn.

[1] This product is available under the trade name "Oilzo" from The G. F. Harvey Company, Saratoga Springs, N. Y.

the rest consisting of the glycerides of saturated fatty acids.[2] A partly ozonized olive oil can thus be best described as a solution of triolein ozonide in olive oil, if one disregards the small amount of trilinolein ozonide which may be present. In this and the following paper the product will be designated simply as ozonized olive oil.

It shows that the decomposition can proceed in two ways, and that water plays an essential role in one of them. The final compounds are acids, aldehydes, organic peroxides, and hydrogen peroxide. Quantitative data for the rate of decomposition of ozonized olive oil are given in Tables I and II.

TABLE I.—SPONTANEOUS DECOMPOSITION OF OZONIZED OLIVE OIL

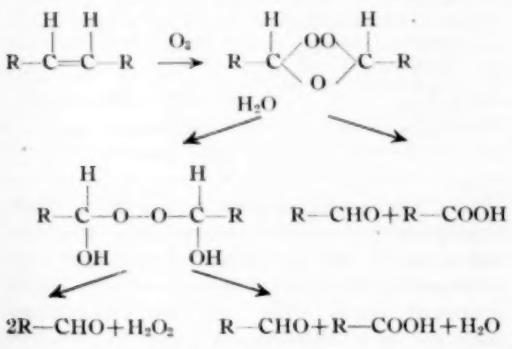
Sample Kept at	Active Oxygen in % (w/w)			Free Acids in Millimol./Gm.		
	Untreated		Dried	Untreated		Dried
	2°	22°	22°	2°	22°	22°
0 wk.	1.37	1.37	1.37	0.71	0.71	0.71
6 wk.	1.32	1.21	1.22	....	....	....
13 wk.	1.29	1.04	1.18	1.07	1.16	1.36
21 wk.	1.27	0.92	1.10	....	....	....
31 wk.	1.24	0.79	1.06	1.44	1.92	2.02

TABLE II.—AMOUNT OF ACTIVE OXYGEN IN OZONIZED OLIVE OIL AND OF HYDROGEN PEROXIDE IN THE AQUEOUS PHASE AFTER 24 HOURS' CONTACT AT 22°

Sample No.	Active Oxygen in % (w/w)			Hydrogen Peroxide, Mg. %	
	1	2	3	1	3
Original Concentration	0.77	0.80	1.41	..	..
After Contact with:					
Distilled water	0.82	0.90	1.49	10.2	11.2
0.1 *M* $NaHCO_3$	0.71	0.89	1.33	4.6	6.5
0.1 *M* NaH_2PO_4	0.81	0.84	1.55	9.8	9.9
Ringer solution	..	0.79	..	..	..

Ozonized olive oil is an almost colorless oil which starts to solidify at a temperature, below 10°. It is very soluble in chloroform, ether, and petroleum ether, and, unlike olive oil, fairly soluble in alcohol and acetone. The surface tension of ozonized olive oil is quite low, approximately 30 dynes/cm.

The structural formula and the decomposition of aliphatic ozonides has been the subject of numerous investigations and controversies, a detailed summary of which is given by Long (13). In a greatly simplified form it can be schematically written as follows:



[2] Other unsaturated compounds, like arachidonic acid, are present only in traces.

EXPERIMENTAL

Due to the complexity of the decomposition reaction no simple analytical procedure for the evaluation of ozonized olive oil is known. For practical purposes an iodometic procedure has been adopted with the results expressed in per cent by weight of active oxygen.

An amount of from 0.1 to 0.2 Gm. of ozonized olive oil is weighed in a little dish, transferred to a 250-ml. Erlenmeyer flask, and dissolved in 10 ml. of chloroform. To this solution is added a mixture of 38 ml. of glacial acetic acid and 2 ml. of a 50% aqueous potassium iodide solution. The flask is closed with a glass stopper and kept with occasional shaking for exactly forty-five minutes in a dark place. After this time, 75 ml. of water are added and the liberated iodine is determined by titration with 0.02 *N* sodium thiosulfate solution (1 ml. = 0.16 mg. O.)

If this method is followed exactly, it will give reproducible results. If one calculates the concentration of the ozonide on the basis that each mole of active oxygen is equivalent to one mole of ozonide one finds only about 70% to 80% of the ozonides present. The reason for this deficiency is partly due to the fact that the reaction has not reached the end point after forty-five minutes but proceeds very slowly for another sixty to ninety minutes. More important, however, is the fact that in the presence of glacial acetic acid part of the ozonide will be split

directly into an aldehyde and an acid without liberation of iodine.

Ozonized olive oil will slowly decompose due to the inherent lability of the triolein ozonide molecule. This decomposition cannot be prevented completely but it can be retarded by removing all moisture from the finished product and storing it at low temperature. An example is given in Table I which contains also values for the amounts of free acids present under the different conditions.

It is interesting to note that the formation of free acids is relatively little influenced by the storage temperature and is not affected by the moisture present.

Since ozonized olive oil is intended only for topical application where moisture is always present, the decomposition in this case is of particular interest and was studied in the following manner:

Ten milliliters of distilled water or 0.1 *M* solution of sodium bicarbonate or monosodium phosphate (*p*H 8.2 and 4.5, respectively) in a small (25-ml.) beaker were completely covered with ozonized olive oil (approximately 0.4 Gm.). The samples were then kept at 37° for twenty-four hours. After this time, the amount of available active oxygen in the oil was determined in the above described manner and also the amount of hydrogen peroxide in the aqueous phase. The average results of a number of experiments are summarized in Table II.

At first it seemed somewhat paradoxical that, after prolonged contact with water, the ozonized oil should yield more active oxygen than it showed originally. The explanation is to be found in the different ways in which the ozonides can be decomposed. It had been indicated that glacial acetic acid can split triolein ozonide directly into an aldehyde and an acid without the liberation of active oxygen in any form. This means that, in the standard procedure of analysis, a certain amount of the ozonide will not react with hydriodic acid and that, therefore, the obtained results are too low. If, however, the oil has been in contact with water for a prolonged period of time, part of it has already been decomposed with the formation of per-compounds. If the standard method of analysis is then applied to this already partly decomposed product, the per-compounds will hydrolyze quantitatively while the remaining ozonides will decompose to the same extent as before. Thus, a simple calculation of the reaction equilibrium proves that a larger amount of active oxygen should be found if the oil was in contact with water before the analysis was made.

Table II contains also values for the amount of active oxygen in the aqueous phase expressed in terms of hydrogen peroxide. As can be expected, the decomposition of both the oil and hydrogen peroxide proceeds at a somewhat faster rate if the *p*H of the solution is shifted to the alkaline side.

The above explanation, that ozonized olive oil after prolonged contact with water shows an apparently greater concentration of active oxygen than the original sample, is also supported by another observation. The kinetics of the reaction employed in the analytical procedure indicate that it is neither of the first nor of the second order. This means that the results are affected by some factors other than the concentration of either ozonide or water. The glacial acetic acid and hydriodic acid are present in such excess that their influence upon the rate of the reaction can be neglected. One must, therefore, conclude that several reactions proceed at the same time.

The experiments which are summarized in Table II give some information as to the rate of decomposition of ozonized olive oil in contact with water and at different *p*H. There are, however, several other factors which, in the practical application, might influence this decomposition. Since the chemical structure of the ozonized oil still resembles that of fats, the possibility of an enzymatic cleavage was of importance. Furthermore, since the product is recommended as a wound dressing, the question arose whether or not surface active forces on cell membranes and tissue surfaces might influence the reaction.

Table III contains a summary of experiments similar to those described above where the water was replaced by hemolyzed blood, coagulated blood, serum, or minced muscle tissue from healthy rats. Of these substances, coagulated blood and serum do not alter appreciably the amount of available active oxygen remaining in the oil while hemolyzed blood, and especially muscle tissue, decrease this amount clearly. This becomes still more evident if one considers that the *p*H in both cases is slightly below 7 and the most comparable figures are those obtained with distilled water where the detectable amount of active oxygen had shown an increase. The results thus indicate clearly that under these conditions the decomposition of ozonized olive oil is actually accelerated by contact with either hemolyzed blood or muscle tissue.

TABLE III.—AMOUNT OF ACTIVE OXYGEN IN % (*w/w*) IN OZONIZED OLIVE OIL AFTER 24 HOURS CONTACT WITH BIOLOGICAL MATERIAL

Sample No.	1	2
Original Concentration	0.77	0.80
After Contact with:		
Distilled water	0.82	0.90
Hemolyzed blood	....	0.72
Coagulated blood	....	0.80
Serum	....	0.78
Muscle tissue	0.63	....

Since it is possible that the final products of the decomposition of ozonized olive oil, other than nascent oxygen or hydrogen peroxide, might have some pharmacological action of their own, they shall be mentioned briefly. The following list contains under 1–4 the 2 aldehydes and 2 acids which will be formed upon the decomposition of triolein ozonide. Since olive oil contains up to 10% linoleic acid which

is also able to form an ozonide, the corresponding decomposition products are included under numbers 5-9. In this tabulation, the possible existence of glycerylesters has been disregarded.

1. Pelargonaldehyde
2. Pelargonic acid
3. ω-Formylcaprylic acid
4. Azelaic acid
5. Caproaldehyde
6. Caproic acid
7. Malonaldehyde
8. Formylacetic acid
9. Malonic acid

DISCUSSION

All these observations have one fact in common, namely, that certain saturated mono- and dibasic aliphatic acids can have a stimulating effect on living cells and several such acids are formed when ozonized olive oil is hydrolyzed in the presence of water.

The implications to the present study are obvious. Several clinical and laboratory observations have indicated that ozonized olive oil promotes the normal wound healing process. The above-mentioned investigations could give one possible explanation of this observed action. In addition, ozonized olive oil also has considerable germicidal activity which will be described in a subsequent paper.

TABLE IV.—STABILITY OF VARIOUS OZONIZED OILS

Ozonide of	Active Oxygen: Initial Concentration in % (w/w)	Active Oxygen: In % of Initial Concentration after 8 Wk. at 2°	22°	35°	Free Acids in Millimole./Gm.: Freshly Ozonized	After 8 Wk. at 22°
Olive oil	3.52	92	82	60	0.71	1.17
Corn oil	4.78	88	77	48	....	....
Peanut oil	7.53	100	92	44	2.20	3.73
Peanut oil	3.99	100	95	60	1.13	2.35
Persic oil	4.24	90	72	46	0.74	1.09
Triolein	6.86	91	80	58	....	....
Ethyl oleate	9.71	95	85	46	9.02	13.34
Ethyl oleate	3.78	95	88	58	3.75	3.92

All these compounds have been found actually as decomposition products of oleic acid ozonide and linoleic acid ozonide by various investigators (14). Compounds 1, 2, 5, and 6 belong to the group of saturated fatty acids and their aldehydes while the others are dibasic acids and their aldehydes. This distinction is important because these 2 groups have quite different biochemical and physiological properties.

Kisch (15) studied the influence of the sodium salts of low molecular fatty acids on cell metabolism of different tissues, and found that they provoke a slight increase. However, his experiments went only as far as oenanthic acid. D'Alessandro and Petrucci (16) found that several fatty acids, among them pelargonic acid, will increase considerably the glycolytic ability of red blood cells. Mention should also be made of the observation by Carruthers concerning the inhibiting action of heptyl aldehyde on carcinogenic substances (17).

There are several publications with regard to specific actions of dibasic acids. J. H. Mueller (18) was able to isolate from liver extract a substance which stimulated the growth of diphtheria bacilli in concentrations as low as 1 part in 200 million. He could identify this substance as pimelic acid which is a lower homolog of azelaic acid. He also tested azelaic acid and other simple dibasic acids, but found none effective. English, Bonner, and Haagen-Smit (19) in their study of the so-called wound hormones of plants found that saturated dibasic acids with 8 or more carbon acids, which include azelaic acid, are able to induce renewed growth in mature plant cells.[3]

The fact, that the decomposition products of ozonized olive oil are acids or of an acidic character, should not prove objectionable to the use of this compound in wounds. It is known that the wound secretion must be acid before the proper healing process can take place (20), because an acid reaction offers better resistance to invading organisms. Some investigators even believe acidity acts as a stimulus to formation of primary mesoblastic tissue and a growth of granulation and accelerated wound healing (21).

In addition to olive oil several tests were made with other vegetable oils (corn oil, peanut oil, persic oil) as well as with synthetic products (ethyloleate and triolein). The formation of the ozonides proceeded equally well in all cases. There are some variations in the stability but they are not significant (Table IV). Of some importance, however, is the fact that the disagreeable odor which the ozonides of vegetable oils develop in time was least offending in olive oil. The

[3] These substances should not be confused with plant growth substances which only accelerate the normal development and growth.

ethyloleate ozonide was quite different. The odor is rather inconspicuous and the product is noticeably less viscous than the other investigated ozonides.

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Organic Ozonides as Chemotherapeutic Agents. II. Antiseptic Properties*

By GEORG CRONHEIM†,‡

The germicidal properties of triolein ozonide in the form of ozonized olive oil have been investigated. Ozonized olive oil *in vitro* is bactericidal to *Staphylococcus aureus, Staphylococcus albus,* and *Streptococcus hemolyticus* and fungicidal to *Trichophyton mentagrophytes, Trichophyton purpureum, Microsporon audouini,* and *Microsporon lanosum.* Against *Monilia albicans* it is only fungistatic.

IN A PREVIOUS PAPER (1) it had been shown that ozonized olive oil decomposes in the presence of water into peroxidic compounds including hydrogen peroxide as well as aliphatic aldehydes and acids. Since peroxides are known to have antiseptic properties, and since recent studies by Keeney and co-workers (2) have shown the fungicidal and fungistatic action of various fatty acids, such as propionic, caprylic, and undecylenic acids, it seemed of interest to investigate the action of ozonized olive oil on bacteria and fungi. Some experiments about the bactericidal properties of ozonized olive oil have been reported by Stevens (3).

The following organisms were included in the present study: *Staphylococcus aureus, Staphylococcus albus, Streptococcus hemolyticus, Monilia albicans, Trichophyton mentagrophytes, Trichophyton purpureum, Microsporon audouini,* and *Microsporon lanosum.* The procedures employed were the agar-plate-cup and the agar-plate method using nutrient agar, blood agar, blood agar base, or Sabouraud's agar. The results were evaluated by measuring the width of the clear agar zones from the cup to outer margin of the zone.

EXPERIMENTAL

The action of ozonized olive oil on the investigated bacteria is definitely bactericidal (Table I). Control experiments with both fresh olive oil[1] and triolein showed neither bacteriostatic nor bactericidal action.

As can be expected, the bactericidal action will be influenced to a certain extent by the surrounding medium. If there are substances present which will react easily with active oxygen, the bactericidal action of the oil will be reduced. This can be visibly demonstrated in blood agar plates where a colorless zone of about 1 mm. is always formed on contact with ozonized olive oil.

The relationship between the amount of available active oxygen and the bactericidal effect is shown in Table II. From these figures it can be seen that a partly ozonized olive oil is the most suitable product for topical use. It combines the greatest bacteri-

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† Present address: The S. E. Massengill Company, Bristol, Tenn.
‡ The author is greatly indebted to Mrs. D. C. Deuel for aid in this work.

[1] The fact that olive oil occasionally shows a weak bacteriostatic and sometimes even bactericidal effect is due to the presence of oleic acid peroxides which are formed on prolonged exposure to air. These peroxides can be detected and estimated in the same manner as ozonides.

TABLE I.—AGAR-CUP-PLATE TESTS USING 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN

Organism	Zone in Mm. after 24 Hr. Using—Nutrient Agar	Blood Agar Base	Blood Agar	Subplating Tests—Total No.	Showing Growth	Showing No growth
Staph. aureus	8–9	6–7	2	18	1	17
Staph. albus	6–7	...	...	10	0	10
Strept. hemol.	10–12	5–8	2–3	27	6	21

cidal power with the deepest penetration. Higher concentrations of triolein ozonide are of no advantage because the oil becomes too viscous while lower concentrations are less effective.

The experiments in the bottom line of Table II were made with a sample of ozonized olive oil which was about two years old and had lost more than half of its original active oxygen content. The amount of decomposition products had correspondingly increased. The figures show that the bactericidal action of the sample is weaker than that of freshly made oil with the same active oxygen content. This indicates that decomposition products do not contribute noticeably to the bactericidal action of ozonized olive oil.

It is obvious that ozonized olive oil does not act instantaneously upon contact like the protein precipitating antiseptics but requires some time for the decomposition of the ozonide and liberation of active oxygen before affecting the organism. In order to test this time factor, small pieces were cut out from the innermost and outermost part of the clear agar zones and were subplated separately. The results shown in Table III are conclusive. The inner zone where the oil penetrated first and had the longest time to act upon the bacteria, was completely sterile, while in the outer part of the clear agar zone the action was only bacteriostatic. The same table also shows that the number of organisms present is an additional factor affecting the action of ozonized olive oil, as evidenced by the varying widths of the zones.

In considering an antiseptic from a practical clinical standpoint, it is important to know whether the agent is able to kill bacteria after they had a chance to grow and multiply. In order to test for this condition, agar plates containing bacteria were incubated for a given time before the ozonized olive oil was added. The results (Table IV) show a very definite though decreasing effect up to a preincubation time of six hours.

TABLE II.—AGAR-CUP-PLATE TESTS USING 2 AND 4 DROPS, RESPECTIVELY, OF OZONIZED OLIVE OIL CONTAINING VARIOUS AMOUNTS OF ACTIVE OXYGEN

Material	Active Oxygen, %	Zone in Mm. after 24 Hr.—*Staph. aureus* 2 Drops of Oil	*Staph. aureus* 4 Drops of Oil	*Staph. albus* 2 Drops of Oil	*Staph. albus* 4 Drops of Oil
Freshly prepared ozonized olive oil	2	...	13–16	...	10–12
	1	10–11	13–16	10–12	16–17
	0.5	6–8	8–10	2–4	6–8
	0.25	2	4–5	1	2–3
	0.125	1	1–2	0	1
2-year-old sample	0.4	2	3–4	1–2	3–4

TABLE III.—AGAR-PLATE-CUP TESTS USING 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN

24-Hr. Broth Culture of *Staph. aureus*	Zone in Mm. after 24 Hr.	Growth in Subplating Tests Samples Taken From Innermost Part of Clear Agar Zone[a]	Outermost Part of Clear Agar Zone[a]
Undiluted	6–7	−, −, −	+, +, +
Diluted 1:10	8–9	−, −, −	−, +, +
Diluted 1:100	11–12	−, −, −	+, +, +

[a] Each + or − sign represents the result of a separate subplating test.

TABLE IV.—AGAR-PLATE-CUP TESTS IN WHICH 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN WERE ADDED AFTER VARYING PREINCUBATION TIMES

Preincubation time, Hr.	Zone in Mm. 24 Hr. after Adding Ozonized Olive Oil—*Staph. aureus*	*Staph. albus*	*Strep. hemol.*
1	4	4	3
2	4	3	...
3	4	2–3	2–3
4	3–4	2	1
6	1–2	0	0
8	0	0	0

The fungi which were tested in this investigation are *Monilia albicans, Trichophyton mentagrophytes, Trichophyton purpureum, Microsporon audouini* and *Microsporon lanosum.* The first three were selected because they are some of the most common organisms in epidermophytosis (4), while the latter two are the causative agents in ringworm of the scalp. The results are summarized in Table V.

Under the tested conditions, ozonized olive oil has a strong fungicidal action against *Trichophyton purpureum, Trichophyton mentagrophytes, Microsporon audouini,* and *Microsporon lanosum.* Against *Monilia albicans* it has only fungistatic action.

Since the growth rate of the fungi used in these experiments is comparatively low, the time effect might be of considerable importance. Therefore, a series of experiments was made like those with bacteria. Agar plates containing the fungi were incubated until an abundant growth was present. Then the plates were covered completely with ozonized olive oil, and incubated for a period ranging from twenty-four to seventy-two hours. After this time, samples were taken out, freed from the oil, and transferred to new sterile plates. The results

TABLE V.—AGAR-PLATE-CUP TESTS USING 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN.

Organism	Incubation Time, Hr.	Incubation Temp., °C.	Zone, Mm.	Subplating Tests[a]
Monilia albicans	48	20	4–5	+ + +
				+ + +
	48	20	3–4	+ + +
				+ + +
	48	20	3–4	+ + +
				+ + +
Trichophyton mentagrophytes	168	20	16–18	− − −
				− − −
	96	25	18–23	− − −
				− − −
	72	25	20–24	− − −
				− + +
	72	25	16–20	− − −
				− + +
Trichophyton purpureum	240	20	19–24	− − −
				− − −
	168	25	18–23	− − −
				− − −
	120	25	12–17	− − −
				− − −
	72	25	14–16	− − −
				− − −
Microsporon audouini	96	35	18–23[b]	− − −
				− − −
	96	35	22–26	− − −
				− − −
Microsporon lanosum	96	35	8–11[b]	− − −
				− − −
	96	35	11–15	− − −
				− − −

[a] Each + or − sign represents the results of a separate subplating test. Samples for the upper 3 tests of each series were taken from the innermost and those for the lower 3 tests from the outermost part of the clear zone.
[b] Only 2 drops of ozonized olive oil used.

as shown in Table VI are again very uniform. The ozonized olive oil will, under these conditions, kill completely all the investigated fungi with exception of *Monilia albicans*. Control samples in which olive oil was used instead of the ozonized oil showed in all cases abundant growth of the organism.

DISCUSSION

From the foregoing experiments, it is apparent that ozonized olive oil has considerable germicidal potency. Although it shares this property with a great number of other chemical compounds, it is quite unique in various respects.

Triolein ozonide itself is lipoid soluble while its decomposition products are water soluble. In addition it has a low surface tension and does not dry out thus preventing any caking or crusting if applied topically. Therefore, one can expect that ozonized olive oil is able to penetrate to places which are inaccessible to many other antiseptics. It is apparently this combination of physical and chemical properties which accounts to a large extent for the numerous beneficial clinical results some of which have already been reported [Greenberger and Helfert (5), Barrows (6), Sharlit (7)].

It has been mentioned that the bactericidal action of ozonized olive oil is due mainly to the active (nascent) oxygen which is formed on decomposition of the ozonides. Undoubtedly, this active oxygen plays also an important role in the fungicidal activity of this material. In addition, however, one must also consider two of the decomposition products of ozonized olive oil, *viz.*, pelargonic and caproic acid. The latter has been shown by Keeney, *et al.* (2), to have fungistatic and fungicidal properties against a variety of organisms. More important because it is pres-

TABLE VI.—AGAR PLATES COVERED WITH OZONIZED OLIVE OIL AND OLIVE OIL, RESPECTIVELY, AFTER VARYING PREINCUBATION TIMES

Organism	Incubation Time in Hours Before Adding Test Substance	Incubation Time in Hours After Adding Test Substance	Growth in Subplating Tests[a] When the Substance Was Ozonized Olive Oil	Growth in Subplating Tests[a] When the Substance Was Olive Oil
Monilia albicans	24	24	+ + +	+ + +
	24	48	+ + +	+ + +
	24	72	+ + +	+ + +
Trichophyton mentagrophytes	48	24	− − −	+ + +
	48	72	− − −	+ + +
Trichophyton purpureum	72	24	− − −	+ + +
	72	48	− − −	+ + +
	72	72	− − −	+ + +
Microsporon audouini	96	24	− − −	+ + +
	96	48	− − −	+ + +
	96	72	− − −	+ + +
Microsporon lanosum	96	24	− − −	+ + +
	96	48	− − −	+ + +
	96	72	− − −	+ + +

[a] Each + or − sign represents the results of a separate subplating test.

ent in greater quantities is pelargonic acid. In very recent investigations Rothman and co-workers (8) have found that this acid is a normal constituent in hair fat of adults while it is apparently lacking in children. Furthermore, pelargonic acid has a strong fungicidal activity against *Microsporon audouini*. Based on these findings the authors postulate that the absence of the ringworm of the scalp in adults is due to the appearance of pelargonic acid in hair fat during adolescence.

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Microscopic Description of the Antihistamine Substances Benadryl and Pyribenzamine*

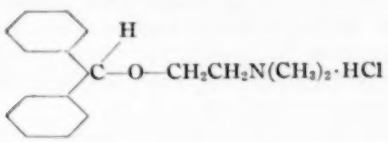
By GEORGE L. KEENAN†

The optical crystallographic properties of benadryl and pyribenzamine have been described, together with significant microchemical reactions with platinic chloride reagent.

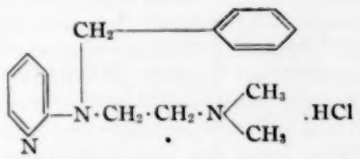
BENADRYL and pyribenzamine have both aroused considerable interest in clinical medicine as antihistamine substances. It is known that histamine is normally present in most of the organs which display allergic manifestations and its presence accounts for many of the symptoms of allergic reactions. Considerable work has been done and reported (1–4) on the effects of a series of compounds with respect to their interference with the action of histamine.

The purpose of this report is to place on record the results of a microscopic study of these two compounds, applying the technic of the immersion method as well as microchemical tests.

Benadryl[1] is β-dimethylaminoethyl benzhydryl ether hydrochloride:



Pyribenzamine hydrochloride[2] is N′-pyridyl-N′-benzyl-N-dimethylethylene diamine hydrochloride:



Both of these compounds are suitable for optical and microchemical study, showing many distinctive features that are useful for

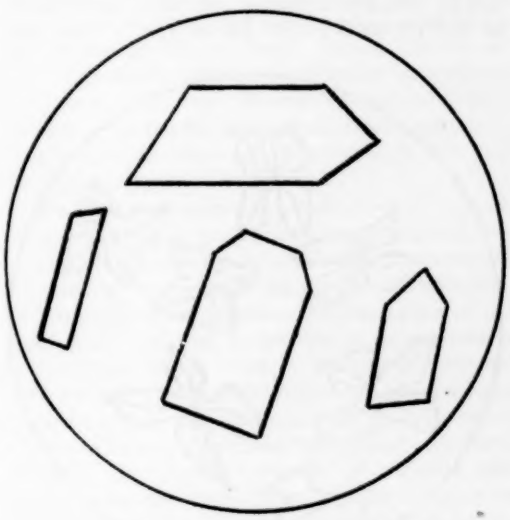


Fig. 1—Benadryl (typical habits).

* Received Jan. 8, 1947.
Presented to the A. A. A. S., Boston meeting, December 28, 1946.
† Formerly Microanalyst, Food & Drug Administration; present address, Strongsville, Ohio.
[1] Furnished through the courtesy of Parke, Davis & Company, Detroit, Mich.
[2] Furnished through courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

diagnosis. For the optical crystallographic study, the immersion method with the polarizing microscope was used. The microchemical tests were made by dissolving the respective compounds in a suitable menstruum, then drawing in a drop of the appropriate reagent. For the significant microscopic characters, the optical crystallographic constants first will be given, followed by a description of the microchemical tests.

EXPERIMENTAL

Benadryl.—Benadryl consists of a white crystalline substance, quite soluble in water and alcohol and stable under ordinary conditions.

In ordinary light: Colorless, most frequently in six-sided plates with lengthwise cleavage (Fig. 1).

Refractive indices: $\alpha = 1.602$, $\beta = 1.625$, $\gamma = 1.630$, all ± 0.002. Most plates show α lengthwise and β and γ crosswise (Fig. 2).

In parallel polarized light (crossed nicols): Extinction is parallel and the sign of elongation is negative.

In convergent polarized light (crossed nicols): Biaxial but only flash interference figures are shown. Optic sign: (−).

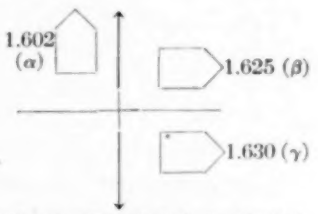


Fig. 2—Benadryl (orientation).

Microchemical Test.—A significant microchemical test for benadryl is platinic chloride reagent (5 Gm. of $H_2PtCl_6 \cdot 6H_2O$ in 100 ml. of H_2O). The most convenient method for making the test is by dissolving approximately 0.5 mg. of the material in a drop of water on an object slide, then drawing in a drop of the platinic chloride reagent. Gradually X-shaped aggregates of very thin blades or leaf-like structures are produced. These blades show a line passing through the center, the general appearance of the structure simulating that of a leaf with midrib. Due to the thinness of the blades, the polarization colors are not at all brilliant (Fig. 3).

Fig. 3—Benadryl with Platinic chloride.

Pyribenzamine.—Pyribenzamine hydrochloride consists of a white, crystalline powder, the habit of the fragments not being significant. It is very soluble in water and when crystallized from a concentrated drop on an object slide shows many rectangular plates and prisms exhibiting striking interference colors with crossed nicols.

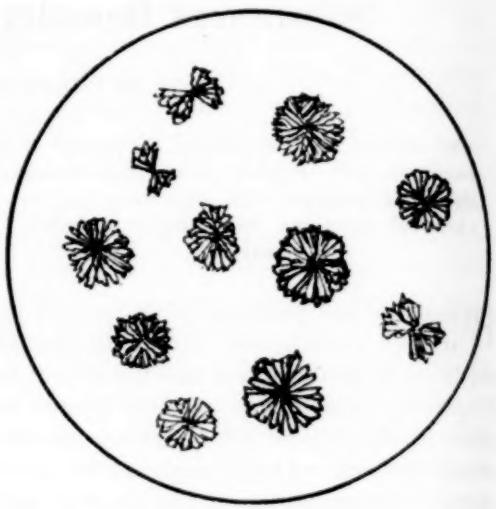


Fig. 4—Pyribenzamine with Platinic chloride.

Refractive indices: $\alpha = 1.580$, $\beta = 1.655$, $\gamma = 1.705$, all ± 0.002. All of the indices are readily found on the substance.

In parallel polarized light (crossed nicols): It is characteristic of the substance that many fragments do not extinguish sharply.

In convergent polarized light (crossed nicols): Biaxial, optic axis figures being commonly shown. Optic sign (−).

Microchemical Test.—With platinic chloride reagent, pyribenzamine forms dense rosettes and sheaves of small rods or blades. The reagent is drawn into the test-drop (about 0.5 mg. in a drop of water) on an object slide, whereupon the characteristic aggregates form (Fig. 4).

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Studies on Alcohol-Soluble Fungistatic and Fungicidal Compounds. I. Evaluation of Fungistatic Laboratory Test Methods*,†

By KURT A. OSTER and MILTON J. GOLDEN

The two principal methods for the determination of fungistasis have been compared and their limitations noted. From the dosage response curves gained by this study a new expression is suggested, the "activity coefficient," to denote degree of fungistatic activity exhibited by a substance. Ten compounds extensively employed in the therapy of dermatophytosis have been tested with *Trichophyton mentagrophytes*, and their relative potencies have been determined.

BURLINGAME and Reddish in 1939 (1) brought the subject of laboratory testing of fungistatic and fungicidal substances up to that date and suggested a new test method, following in principle the serum agar-cup-plate technique described in the U. S. Department of Agriculture Circular No. 198 (2). However, aside from the statement that their findings ran closely parallel with clinical observations, this paper made no mention of actual controls or of any limitations in their method. Schamberg and Kolmer (3) had suggested in 1922 a fungistatic test method to which Burlingame and Reddish objected, claiming that it did not simulate clinical conditions.

In 1945 the Council on Pharmacy and Chemistry of the American Medical Association issued a report (4) in which it was declared urgent that a standard test for fungistatic and fungicidal substances be established. Use of the fungicidal test known as the phenol coefficient test for disinfectants and antiseptics, as modified by the American Public Health Association subcommittee, was suggested. However, it was noted that the test is meaningful only for surface growth, such as "testing the action of fungicides on floors and the like." Furthermore, it was stated that the problem of testing nonwater-soluble products presents even greater difficulties.

In view of the unsettled status of these test procedures, it was considered a desirable undertaking to subject to critical laboratory evaluation the principally used alcohol-soluble compounds for the therapy of foot dermatophytosis and also the existing test methods by which the comparative fungistatic potencies of these compounds are assayed.

METHODS

Following are the outlines of the methods used:

Burlingame and Reddish (1).—Solutions are tested by the agar-cup-plate technique. The serum agar is prepared by adding 2 cc. of sterile normal horse serum to 18 cc. of Sabouraud's agar, which has been melted and cooled to 40°. The agar is poured into a 9-cm. Petri dish, and, when hardened, the entire surface of each plate is streaked with a ten-day-old culture of the test organism. A 2-cm. cup is cut from the center of the plate with a sterile cork borer, and approximately 0.8 cc. of the test fluid is pipetted into the cup. The plates are then covered with unglazed clay tops to prevent condensation and incubated for five days. The fungistatic activity of a given compound is indicated by a cleared zone around the cup. Solvent controls with 95% alcohol accompanied each series of determinations. The fungistatic effect of the alcohol in this test method was found to be minimal.

Modified Burlingame and Reddish (5).—A modification of the agar-cup-plate technique has been suggested by substituting incorporation of a ten-day-old conidial suspension in the agar for the procedure of streaking a fungus culture on the surface of the agar plate. A 1-cc. suspension of approximately five million spores from a ten-day-old culture, counted in a haemocytometer and diluted with saline, is thoroughly mixed with 15 cc. of Sabouraud's agar, which had been melted and cooled to 40°. An alcoholic solution of the test substance is pipetted into a 2-cm. cup, cut and removed from the center of the hardened agar. The plates, covered with porous clay tops, are incubated for 96 hours, at which time the clear zone around the cup, indicating inhibition of growth, is measured.

* Received March 29, 1947, from the Research Laboratories of McKesson & Robbins, Incorporated, Bridgeport, Conn.

Presented in part before the Division of Biological Chemistry of the American Chemical Society at its semiannual meeting, Atlantic City, N.J., April, 1946.

† A recent publication by Hillegas and Camp (10) on the testing of fungicides insoluble in water came to our attention after the initiation of this work. These authors express ideas on the comparative measurement of fungistatic compounds which are similar to those propounded in our present study. However, their suggested "penicup" method for determining fungistasis was not reproducible in our hands.

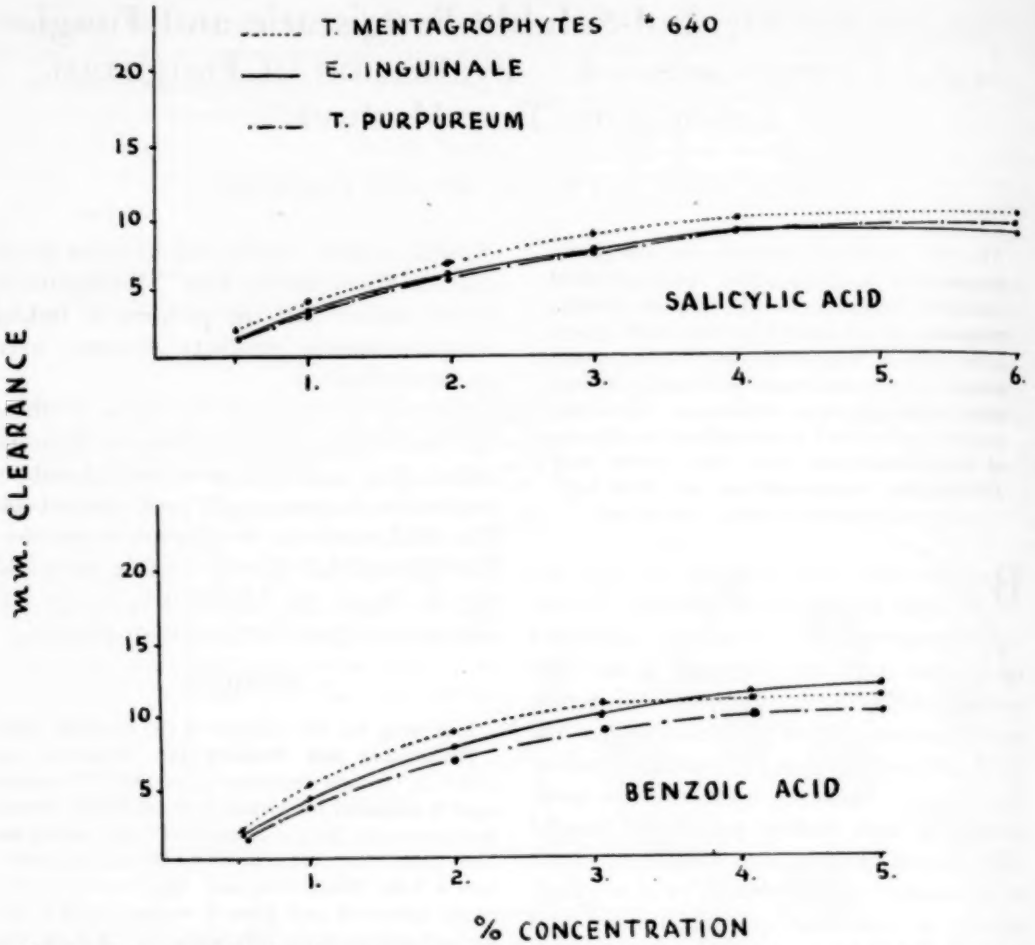

Fig. 1.—Dosage and fungistatic response curves of three species of fungi with increasing concentrations of salicylic acid and benzoic acid.

Schamberg and Kolmer (3).—This method for determining the fungistatic effect of water-soluble substances has been so modified as to be applicable to alcohol-soluble compounds without violating its underlying principles. In view of the fact that alcohol itself possesses minor fungistatic properties, it was necessary to limit the quantity of alcohol incorporated in the agar to 0.1 cc., an amount which exerted no noticeable fungistasis. Plates were poured, each containing 20 cc. of Sabouraud's agar into which 0.1 cc. of an alcoholic solution of the test substance had been incorporated, thereby diluting by two hundred times the original concentration of the test substance in alcohol. The surfaces of the plates were streaked with a ten-day-old culture of the test organism, and the plates were incubated for two weeks, after which time the presence or absence of growth was noted. The highest dilution of the test material which completely inhibited the growth of fungi after a two-week incubation period was regarded as the critical fungistatic dilution.

The 3 fungi commonly associated with dermatophytosis of the human foot, *Epidermophyton inguinale*, *Trichophyton purpureum* and *Trichophyton mentagrophytes* (*T. interdigitale*, *T. gypseum*), were first tested individually. *T. mentagrophytes* (#640 Emmons) was later chosen as the sole test organism.

The incubator temperature for all tests was 28° ±1°. Stock cultures of the fungus were stored on maltose agar slants at 2–5°. At intervals not longer than one month transfers were made to fresh agar slants, incubated at 28° for at least ten days, and placed in storage at 2–5° until the next transfer period. It was ascertained with the phenol coefficient technique (6) that a spore suspension of a ten-day-old culture of *T. mentagrophytes* was killed after contact with phenol for ten minutes at a concentration of 1:60 and not by a concentration of 1:80.

The following 10 active compounds commonly used in the therapy of dermatophytosis were chosen for study: benzoic acid, salicylic acid, phenol, resorcinol, cresol U. S. P., metacresylacetate, thymol, chlorothymol, propionic acid, and undecylenic acid.[1] Of the 103 marketed athlete's foot remedies with declared formulas, as collected by Underwood,

[1] Undecylenic acid supplied by Dr. L. Reiner, Wallace & Tiernan Products, Inc., Belleville, N. J.

et al. (7), 76 contained as active ingredients from 1 to 4 of these compounds under test.

RESULTS

Using 5 determinations for each organism and for each concentration with the Burlingame and Reddish method, reproducible inhibition zones for the evaluation of the fungistatic action of the above-mentioned compounds have been obtained in a range of critical concentration. The inhibition of growth around the cup was measured in millimeters from the edge of the cup to the periphery of the cleared zone, and averages of the 5 measurements of these radius segments were calculated.

Approximately 400 individual determinations were made with various concentrations of the 10 compounds. It was established that the fungistatic values obtained with the examples of salicylic acid and benzoic acid for the 3 test organisms, *T. mentagrophytes*, *E. inguinale*, and *T. purpureum*, ran closely parallel. In subsequent tests *T. mentagrophytes* (#640 Emmons) was chosen as the representative test organism to save time and to reduce the number of tests. It was assumed that *E. inguinale* and *T. purpureum* would give with the remaining 8 compounds values approximating fairly closely those of *T. mentagrophytes*.

It was observed that the cleared zones exhibited the greatest variations with either very low or very high concentrations of the test substance. Values falling between the 2 extremes never deviated more than ±1 mm. Typical dosage response curves were obtained by plotting the radius segments of clearance against the respective concentrations. These findings are shown in Figs. 1 and 2. The shape of each curve was determined by at least 5 points at various concentrations. In some instances only a

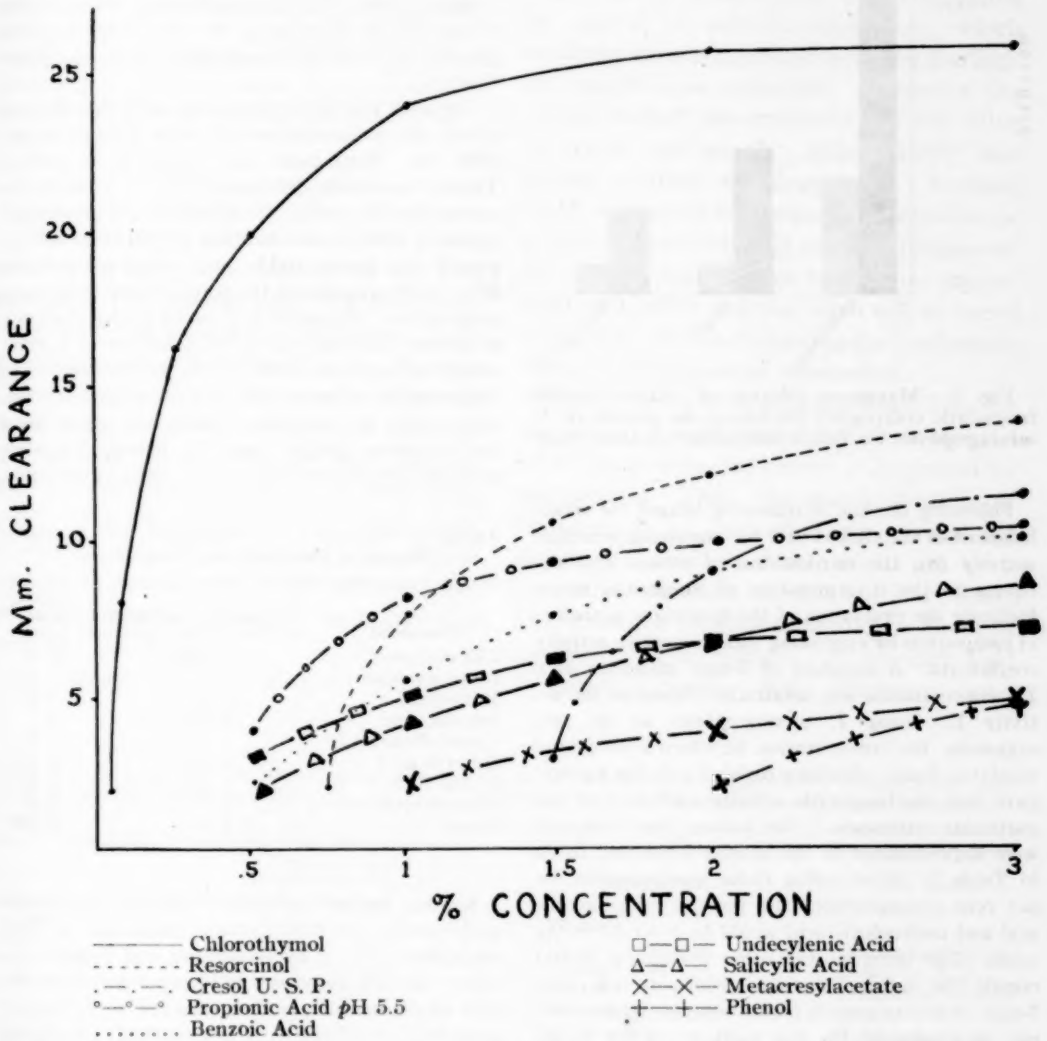

Fig. 2.—Dosage and fungistatic response curves of *T. mentagrophytes* No. 640 with increasing concentrations of various alcohol soluble compounds.

portion of the curves is presented, because values in excess of 3% concentration were eliminated in the illustration. From these dosage response curves it appeared that all the substances tested exhibited a ceiling of activity. Once this ceiling had been reached, an increase in concentration did not induce a corresponding rise in fungistasis.

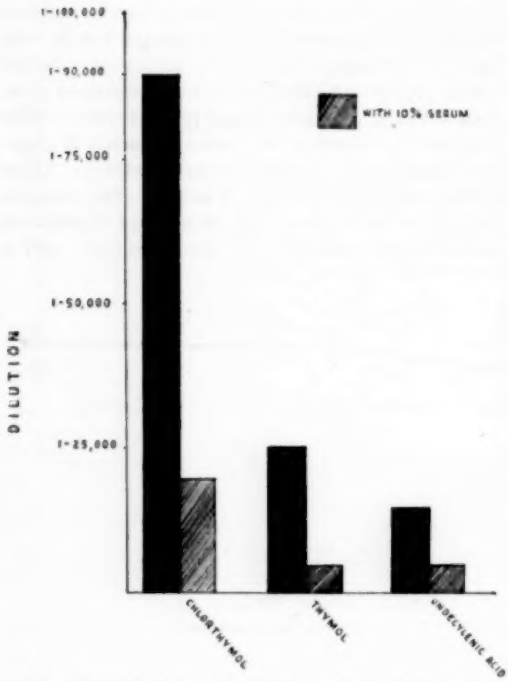

Fig. 3.—Maximum dilution of alcohol soluble fungistatic compounds inhibiting the growth of *T. mentagrophytes* No. 640 on Sabouraud's maltose agar.

Following the line of reasoning behind the establishment of the Oxford unit for expressing penicillin activity (8), the introduction of dosage response curves in the determination of fungistasis might facilitate the evaluation of the fungistatic activities of compounds by expressing their respective activity coefficients. A standard of 5-mm. clearance at a 1% concentration was arbitrarily chosen as the activity 1. Using *T. mentagrophytes* as the test organism, the concentration at which a compound exhibited 5-mm. clearance divided into the figure 1 gave then the fungistatic activity coefficient of this particular substance. The values thus obtained were approximated to the nearest tenth and listed in Table I. Substituting molar concentration for per cent concentration, the position of propionic acid and undecylenic acid would be reversed in the table. The order of the other substances would remain the same. Substances which do not reach 5-mm. clearance even in higher concentrations could not be evaluated by this method. They would also most likely fall short of any therapeutic usefulness.

Using the described modification of the Burlingame and Reddish technique (5), which requires incorporation of a conidial suspension with the agar, gave much the same results with the compounds as encountered with the original Burlingame and Reddish procedure of culture streaking. The zone of inhibiton of growth was slightly larger than that observed with the streaking method. Furthermore, the cleared zone was almost a perfect circle, a result which was not always obtainable with the Burlingame and Reddish technique. However, these advantages were outbalanced by the inherent cumbersomeness of the method, which requires standardization, counting, and continuous availability of a suitably aged spore suspension. This is extremely time consuming, especially in large-scale routine testing of substances. Sealing the cup was found to be unnecessary, provided the agar medium was not cracked, since equal inhibition was obtained with both sealed and unsealed plates. The greater clearance shown by this modification might be due to the absence of serum in the agar medium, which greatly inhibited the fungistatic action of certain chemicals.

The more volatile compounds in our series, thymol, cresol, and metacresylacetate, were difficult to test with the Burlingame and Reddish technique. Thymol was finally eliminated from the series due to unreproducible results. Undiluted metacresylacetate caused a whitish discoloration of the agar, and no growth was demonstrable, even when using sterile filter paper supports for the porous plates to enhance evaporation. Cresol U. S. P. and metacresylacetate in various dilutions up to 3% inhibited to a great extent the surface growth of the Trichophytons, so that with these compounds only subsurface development could be measured. Moreover, when using concentrations greater than 3%, it was impossible to obtain reproducible values with these compounds.

TABLE I.—ACTIVITY COEFFICIENTS OF ALCOHOL-SOLUBLE FUNGISTATIC COMPOUNDS

Compound	% Conc. of Test Solution to Give 5-Mm. Clearance	Activity Coefficient
Chlorothymol	0.0625	16.0
Propionic acid	0.60	1.7
Resorcinol	0.81	1.2
Benzoic acid	0.91	1.1
Undecylenic acid	1.0	1.0
Salicylic acid	1.28	0.8
Cresol U. S. P.	1.58	0.6
Metacresylacetate	3.35	0.3
Phenol	4.0	0.25

Another method generally employed with minor modifications to demonstrate fungistasis is that originally proposed by Schamberg and Kolmer (3), where the test substance is incorporated with the agar medium which is then streaked with the test organism. Findings with this method, comparing the fungistatic activities of the previously mentioned 10 compounds, are charted in Figs. 3 and 4.

This test reveals the outstanding action of chlorothymol, which is fungistatic for *T. mentagrophytes* in dilutions of up to 1:90,000. This substance is followed by thymol, 1:25,000, and undecylenic acid, 1:15,000. The remainder of the compounds displayed no remarkable activity, their fungistatic activities appearing in dilutions of from 1:2500 to 1:1500.

The medium suggested by Schamberg and Kolmer contained no serum. When normal horse serum was added to the medium to give a concentration of 10%, the fungistatic action of 7 of the test compounds was remarkably curtailed. Chlorthymol lost 78% of its fungistatic power, dropping from 1:90,000 to 1:20,000. Only cresol, metacresylacetate, and phenol were not affected by the addition of serum.

Peck and Rosenfeld (9) claimed ascorbic acid to be fungistatic. This statement could not be confirmed by us. It was found that ascorbic acid dissolved in 95% alcohol exhibited no zone of growth inhibition even at a concentration of 2% with the Burlingame and Reddish method. Using the Schamberg and Kolmer method, it did not prevent the growth of *T. mentagrophytes* in dilutions of up to 1:500.

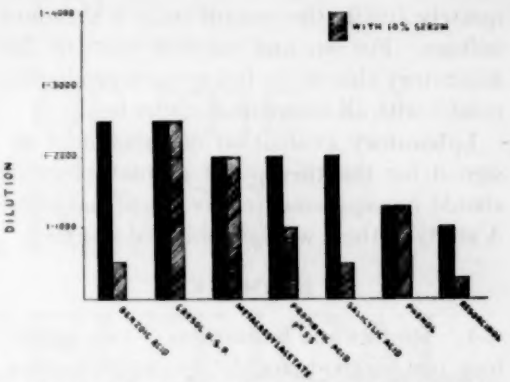

Fig. 4.—Maximum dilution of alcohol soluble fungistatic compounds inhibiting the growth of *T. mentagrophytes* No. 640 on Sabouraud's maltose agar.

DISCUSSION

The need for the establishment of a standard test for fungistatic substances has long been felt. The present study has attempted to evaluate the fungistatic potencies of ten compounds commonly used in the therapy of dermatophytosis. The results observed by utilizing the two principal test methods as suggested by Burlingame and Reddish and by Schamberg and Kolmer were compared. On arranging the ten test compounds in an order of decreasing fungistatic activity, no correlation could be noted between the results obtained with the two methods. See Table II.

The Schamberg and Kolmer procedure proved to be quite helpful in investigating the effects of volatile substances, which could not be accomplished with the Burlingame and Reddish technique. However, the former method demonstrated but one value for each compound; either there was growth or there was fungistasis. Nothing could be learned of eventual penetration or of ceiling of activity. It is the impression of the authors that the two techniques supplement each other and that each will be useful in its own right for evaluating the mechanism of action of fungistatic substances.

A new means of expressing fungistasis by measuring the activity coefficient derived from dosage response curves is suggested by the authors. Thus, the standardization of the activity of one substance against the activity of another, which might act by a completely different mechanism is avoided. The standard conditions would be those of medium and of culture. It appears that *T. mentagrophytes* (#640 Emmons) ade-

TABLE II.—COMPARISON OF TEN COMPOUNDS ARRANGED IN A DECREASING ORDER OF FUNGISTATIC ACTIVITY DERIVED FROM THE BURLINGAME AND REDDISH AND THE SCHAMBERG AND KOLMER METHODS

Burlingame and Reddish (Fig. 2)		Schamberg and Kolmer (Figs. 3 and 4)	
Activity Coefficient	Height of Ceiling of Dosage Response Curve	Without Serum	With Serum
Chlorothymol	Chlorothymol	Chlorothymol	Chlorothymol
Propionic acid	Resorcinol	Thymol	Thymol
Resorcinol	Cresol U. S. P.	Undecylenic acid	Undecylenic acid
Benzoic acid	Propionic acid	Benzoic acid	Cresol U. S. P.
Undecylenic acid	Benzoic acid	Cresol U. S. P.	Metacresylacetate
Salicylic acid	Salicylic acid	Metacresylacetate	Phenol
Cresol U. S. P.	Undecylenic acid	Propionic acid	Propionic acid
Metacresylacetate	Metacresylacetate	Salicylic acid	Benzoic acid
Phenol	Phenol	Phenol	Salicylic acid
		Resorcinol	Resorcinol

quately fulfills the conditions of a standard culture. For one and one-half years in this laboratory this strain has given reproducible results with all compounds under test.

Laboratory evaluation of substances designed for the therapy of dermatophytosis should be supplemented by fungicidal tests. A study of these will be published shortly.

SUMMARY

1. Results and limitations of two laboratory test methods for determining fungistasis, devised by Burlingame and Reddish and by Schamberg and Kolmer, were compared by studying the fungistatic activities of ten alcohol-soluble compounds on cultures of *T. mentagrophytes* (#640 Emmons).

2. The introduction of dosage response curves in determining fungistasis suggested a new expression of fungistatic activity, the activity coefficient.

3. The value of the two methods in the determination of fungistasis has been discussed.

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Book Review

Vitamins and Hormones, Volume IV. Edited by ROBERT S. HARRIS and KENNETH V. THIMANN, Academic Press Inc., New York, 1946. xvii + 406 pp. 14.5 x 23 cm. Price $6.80.

This is the 4th annual review of the literature in the vitamin and hormone field. When the first review was published in 1943 many no doubt wondered at the necessity for reviews published in such diversified fields. More and more, however, it is becoming evident that the two fields overlap. No doubt this will become even more evident as more research is done on the influence of the hormones on intermediary metabolism.

The first review, by Pfiffner and Hogan, is on "The Newer Hematopoietic Factors of the Vitamin B-complex." This is a very interesting review of folic acid, vitamins B_c, M, and other identical or closely related substances having folic acid activity. The authors give a number of ultraviolet absorption curves which show how closely most of these factors are interrelated. The enzyme, Vitamin B_c conjugase, which splits vitamin B_c from its conjugate is also covered. The relationship of these substances to Xanthopterine from butterflies' wings is of considerable historic interest. The authors cite 122 references.

Dr. Howard A. Schneider of Rockefeller Institute summarizes very succinctly "Nutrition and Resistance to Infection." It is here pointed out that nutritional factors may contribute either to susceptibility of, or resistance to, infection and that the action of any specific diet may well be the resultant of these opposed forces.

"Manifestations of Nutritional Deficiency in Infants," by F. W. Clements, is of interest to those working in pediatrics.

No doubt the chapter on "Nutritional Therapy of Endocrine Disturbances," by Morton S. Biskind, will cause considerable comment for its unorthodox point of view. Of equal interest will be the chapter on "The Thyroid and Diabetes," by Bernardo A. Houssay, which may be supplemented by "Thyroactive Iodinated Proteins," by E. P. Reinecke.

To pharmacists probably the most important chapter is on "Methods of Bioassay of Animal Hormones," by Sidney A. Thayer. Dr. Thayer first discusses the principles which should govern biological methods, i.e. the nature of the product, the determination of animal variation, the choice of a suitable standard, and units. This is followed by a short section on the statistical treatment of the data which includes a description of the standard deviation and the meaning of the regression line. This is followed by a more detailed discussion of the bioassays of the gonadotropic hormones, the remaining tropic hormones of the pituitary and the adrenal cortical hormones.—MELVIN W. GREEN.